The pathogenic and toxic effects of the fungus Beauveria bassiana on Manduca sexta larvae

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The pathogenic and toxic effects of the fungus *Beauveria bassiana* on *Manduca sexta* larvae

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"What do you mean by that ?" said the Caterpillar sternly. "Explain yourself !"

Alice's Adventures in Wonderland

Lewis Carroll

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ABSTRACT

Beauveria bassiana has been isolated from over 170 different species of insects, but it is not known as a pathogen of *Manduca sexta*. By comparing the effects of different isolates of *B. bassiana* on *M. sexta* larvae different fungal strategies become apparent.

Two distinct treatments of larvae with spore suspensions, immersion and injection, showed that whilst all twelve tested isolates were pathogenic 72 hours post-injection, few of the isolates were pathogenic by immersion. This suggests that there exists a difference in the ability of different isolates to penetrate the insect cuticle .

Different spore concentrations of all twelve isolates were used to determine the LC_{50} both by injection and immersion. It was shown that sub-lethal treatments reduced weight gain and delayed pupation. Faeces production was monitored and showed a similar pattern to weight gain suggesting that treatment with low spore concentrations might induce a reduction in feeding, or a malaise in the larvae.

The haemolymph of larvae treated by injection with fungal spores showed blastospores at 48 h post-injection and hyphal fragments at 72 h. Treated samples showed a reduction in the number of haemocytes, compared with the control, after 72 h. The haemolymph of immersed insects showed no blastospores or hyphal fragments even at 4 days. The haemocyte level of all immersed insects was similar to that of the control at 1×10^5 per ml.

Beauveria bassiana is known to produce a range of metabolites, some of which are toxic to insects. The filtrate from growth in liquid media of all the isolates was used to treat fifth instar larvae by injection. Early mortality after spore injection was not always echoed by early mortality after filtrate injection from the same isolate.

Although there was variability in pigment production between the isolates, those producing red pigment were not more pathogenic than those which did not. However the isolates which produced a red pigment on agar and in liquid culture filtrate were also able to cause a change in the colour of the larvae from green to red 96 hr after injection

Conidiospores of *Beauveria bassiana* stimulated haemocytic responses in the form of phagocytosis and nodule formation. Fungal filtrate from cultures grown *in vitro* was shown to reduce the incidence of phagocytosis.

Fungal spores of different isolates varied in their ability to stimulate a nodule-forming response by haemocytes. This variation correlated both *in vivo* and *in vitro* with the pathogenicity of the isolate for the larvae.

Heated conidiospores did not stimulate haemocytes to produce nodules.

Although spores from all the isolates promoted nodule production, filtrate from fungal growth *in vitro* of some isolates reduced it. The eicosanoids, indomethacin and dexamethathasone, also reduced the production of nodules and appeared to work synergistically with the fungal filtrate.

Some fungal filtrates reduced the production of haemocytic nodules in a dose-dependent fashion. The effect was not altered by heating the filtrates, but was destroyed by proteolytic enzymes.

Gel electrophoresis showed that the filtrates which were effective in suppressing nodule formation contained peptides, or small proteins, of less than 10kDa. These metabolites were absent in filtrates which did not reduce the formation of haemocytic nodules.

ABBREVIATIONS

Balb/c	-	strain of mouse
° C	-	degrees Celsius
C-Dox	-	Czapek-Dox medium
DABS	-	diaminobenzoline hydrochloride
dex	-	dexamethasone
DF	-	statistical degrees of freedom
dH_2O	-	distilled water
DMAB	-	3(dimethylamine)benzoic acid (for oxalic acid assay)
EDTA	-	ethylenediaminetetraacetic acid
ELISA	-	enzyme-linked immunoabsorbent assay
FITC-WGA		fluoresecin isothiocyanate conjugated wheat germ agar
g	-	gravity acceleration units
g	-	grams
GAG	-	glucosaminoglycan
GIM	-	Grace's insect medium
GR	-	granular haemocyte
h	-	hours
ind	-	indomethacin
kDa	-	kilodaltons (measure of molecular weight)
LC_{50}	-	concentration lethal to 50% of the trial organisms
LT_{50}	-	time to kill 50% of trial organisms
mg	-	milligrams
min	-	minute(s)
ml	-	millilitres
M	-	molcs or molar
MBTH	-	3-methyl-2-benzothiazoline hydrazone (for oxalic acid assay)
nm	-	nanometres (measure of UV wavelength)
O	-	oenocytoid haemocyte
p	-	probability of chance result
PBS	-	phosphate buffer saline
pH	-	measure of acidity/alkalinity
PL	-	plasmatocyte haemocyte

ppm	-	parts per million
PTU	-	phenylthiourea
RH	-	relative humidity
RPMI1640	-	cell culture medium defined by Roswell Park Memorial Institute, Buffalo, NY.
s	-	seconds
SDAY	-	Sabouraud's Dextrose Agar with Yeast Extract
SDS-PAGE		sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SEM	-	standard error of mean
TEMED	-	N,N,N',N'-tetramethyl ethylenediamine
THC	-	total haemocyte count
UV	-	ultraviolet
1d 5L	-	first day fifth instar larval stage
µl	-	microlitre
µg	-	microgram

CONTENTS

Chapter 1

Introduction

1.1 *Beauveria bassiana*, an entomopathogenic fungus

The entomopathogenic fungus *Beauveria bassiana* was first recognised early in the nineteenth century (Bassi 1835). *Beauveria bassiana* has a wide host range and is known as a lepidopteran pathogen. It has been isolated from *Spodoptera frugiperda, Cydia pomonella, Heliothis virescens, Plutella xylostella*, as well as *Bombyx mori* (Humber 1992). It has a large geographic range and isolates have been collected from four continents and many different climatic regions.

Beauveria bassiana was the first fungus to be definitely linked to insect disease when in 1834 Agostino Bassi recognised it as the cause of the white muscardine disease of *Bombyx mori*, the silkworm. The fungus was responsible for the infection and death of commercial silkworms and subsequently for the demise of the French and Italian silk industries in the early 19th century. A study of the diseased silkworms enabled Bassi to understand how fungal disease was transmitted between insects at a time when infection was thought to arise spontaneously.

Manduca sexta, the tobacco hornworm, is a lepidopteran insect pest which, whilst not currently of great commercial importance, has been much studied as a 'model' lepidopteran. It has been used for studies on the mechanisms of fungal pathogenesis, as reviewed by St Leger (1995). Much of this work has utilised the green muscardine fungus *Metarhizium anisopliae* (St Leger, Cooper and Charnley 1988a, Sloman and Reynolds 1993) and there are no records of the isolation of *B. bassiana* from wild *Manduca sexta*.

In the present study, the pathogenicity of various isolates of *B. bassiana* toward *M. sexta* was studied in order to provide information about the interaction between host and parasite, with a view to learning more about mechanisms of pathogenesis.

Entomopathogenic fungi, like other pathogens, need to overcome the defences of their hosts in order to be successful. These defences comprise:-

A Behavioural	—	Insects may avoid or remove fungal spores;
B Integumental	—	The insect cuticle provides a barrier to invasion by fungi;
C Humoral	—	Circulating insect chemicals, either pre-existing or induced, may kill invading fungi;
D Cellular defences	—	Insect blood cells may detect and kill fungal cells once penetration has occurred.

An effective entomopathogen may have a range of strategies and may produce a number of different toxic metabolites once inside the host, but first it must gain entry to the insect. The way in which *Metarhizium anisopliae* overcomes host insect defences has been studied in greater detail than *Beauveria bassiana*. Work with *Metarhizium anisopliae* on *Manduca sexta* larvae (St Leger, Butt, Goettel, Staples and Roberts, 1989) has shown that fungal entry to the host follows a particular sequence of events. Most of this work has used one particular isolate, ME1, and although it is unlikely that all isolates of the fungus follow precisely the same sequence, the intensive study of one particular isolate has been helpful in elucidating the general strategy of the fungus. It is likely that *Beauveria bassiana* follows a similar sequence, but the exact order of events and their relative importance will doubtless differ from the case of *M. anisopliae* ME1.

Using electron microscopy to study the infection by *M. anisopliae* of wireworms Zacharuk (1970) recognised six stages in the infection process. First, fungal conidiospores must germinate. Next the conidia must attach to the cuticle after which, thirdly, a germ tube emerges from the conidiospore and grows for a variable distance before forming an adhesion plate, the appressorium. Appressorial formation is dependent on the cuticle's surface topography and chemistry. The ratio of complex carbon and nitrogen compounds is known to be important at this stage (St Leger *et al.* 1989). Appressorium formation is one of the fundamental events of development in entomopathogenic fungi, without which the penetration peg will not develop and normal entry through the cuticle cannot occur (Clarkson and Charnley, 1996). Although fungal spores could enter an insect via the digestive system, or through the spiracles (McCauley, Zacharuk and Tinline, 1968) the most usual route of entry for pathogenic fungi is by the penetration peg breaching the epicuticle.

Once the penetration peg has reached the procuticle a penetration plate produces hyphae which are able to grow through the epidermis. When the hyphae reach the haemocoel they give rise

to smooth detached hyphal bodies which are able to circulate in the haemolymph before developing into mycelia which in turn may fill the coelomic cavity and thence may invade the remaining host tissues and kill the insect. After the death of the host emergent hyphae appear on the outside surface of the insect. These may sporulate producing more infective spores thus spreading the fungal infection.

Boucias, Hung, Mazet and Azbell (1994) suggest that the entry of *B. bassiana* follows the same sequence of host penetration as *M. anisopliae* and that the hydrophobicity of its outer layers aids attachment to the insect cuticle. In addition the production of a variety of exocellular enzymes by the pre-germinating conidia may aid their attachment to the cuticle or provide a signal for germ tube formation.

According to Pendland, Hung and Boucias (1993), once the fungus has invaded the host the hyphal bodies of *B. bassiana* appear to be different from those cultured *in vitro*. They lack the galactomannan coat and possess a surface coat that either mimics the host components, or is made up of haemolymph proteins, and is thus not recognised by the host insect's haemocytes, and therefore not destroyed. These authors speculate that it is this ability to avoid the host's defences that allows *B. bassiana* to be an effective pathogen of *Spodoptera exigua*, though it should be noted that this mechanism may not occur in other insect hosts or with all *Beauveria* isolates. Finally, the continued evasion of the host's defences may be assisted by the production of toxic metabolites. In the case of *M. anisopliae* these include the well-studied cyclic peptides, the destruxins (St Leger, Cooper and Charnley, 1986). These have a number of adverse effects on the host including paralysis of the skeletal muscles (Samuels, Reynolds and Charnley, 1988b), inhibition of urine formation by Malphigian tubules (James, Kershaw, Reynolds and Charnley,1993) and inhibition of ecdysteroid synthesis in the prothoracic glands (Sloman and Reynolds, 1993). However the effects of the toxins on the immune system are likely to be particularly important in helping to establish mycosis.

In modern times, *B. bassiana* has been used in practical biological control and has been shown to be a useful pathogen against diverse insect pest species including the red fire ant, *Solenopsis invicta*; the wax moth, *Galleria mellonella* ; the European corn borer, *Ostrinia nubilalis* ; the livestock ticks, *Rhipicephalus appendiculatus* and *Amblyomma variegatum;* and the tsetse fly, *Glossina morsitans* (Gupta, Leathers, Elsayed, and Ignoffo, 1994; Sanchez-Pena and Thorvilson, 1995; Kaaya and Munyini, 1995; Lewis, Berry, Obrycki and Bing, 1996; Kaaya,

Mwangi and Ouna,1996).

A considerable amount of work has already been carried out on the pathogenicity of *B. bassiana* towards different insect species (Bidochka, Miranpuri and Khachtourians, 1993; Varela and Morales, 1996; Inglis, Johnson and Goettel, 1996). However the mechanisms by which the fungus affects its host are diverse and much remains to be clarified.

1.2 Invasion of the host

Over thirty years ago Müller-Kögler (1965) appreciated the unique character of the fungal pathogens of insects. Fungi are able to invade their hosts through the integument, whereas pathogenic bacteria and viruses penetrate mainly after ingestion or injury, so fungal invasion is often independent of the uptake of food, or of trauma. The susceptibility of particular insect species to fungal disease may however show variations depending on the condition of the host, biotic factors of the environment and the individual fungal pathogen.

Specific nutritional requirements may restrict the host range of entomopathogens (Charnley, 1989). Those with broad ranges like *Beauveria bassiana* germinate *in vitro* in response to non-specific sources of carbon and nitrogen, though a possible variation exists in the nutritional demands of different isolates. Others, with narrower host ranges, like *Nomuraea rileyi,* respond to specific polar lipids and diacylglycerides (Boucias and Pendland, 1984). The ability to utilise particular components of the cuticle, like the lipids of the epicuticle, as a nutrient source may increase the success of some fungi.

The fungal pathogen may also need to withstand inhibitory factors produced by the host, like those contained in the cuticle of *Heliothis zea* (Smith and Grula, 1982), so fungal penetration is a highly specific activity which is only successful if a particular array of circumstances arises.

1.3 Pathogenicity

Beauveria bassiana is pathogenic to a wide range of insect species and its mode of entry into the host varies. In many insect species *Beauveria bassiana* spores are able to penetrate the cuticle and gain entry to the haemocoel. This ability may be due as much to the composition of the insect cuticle as to the variation between different fungal isolates. Insect cuticle is composed of a series of layers of chitin fibres embedded in a protein matrix. Some of the layers also include lipids and waxes (Jeuniaux, 1984), but the thickness and composition of the layers varies between species and stages of the insect as well as with the region of the insect body. Hence penetration of the insect cuticle depends on the ability of the fungus to produce a range of enzymes.

Samsinákóvá (unpublished, 1968) found that *B. bassiana* produced a chitinase that was active against pure chitin and also against the integument of *G. mellonella* larvae. Later Samsinákóvá, Bajan, Kàlakovà, Kmitowa and Wojiechowska (1977) showed that the ability of *B. bassiana* germinal hyphae to penetrate the host cuticle was directly related to the amount of chitinase produced. They also showed that *in vitro B. bassiana* produced lipase and protease which could contribute to penetration success.

St Leger, Cooper and Charnley (1986) concluded that the proteases and chitinases produced by *Metarhizium anisopliae* and also *Beauveria bassiana* act synergistically in the solubilisation of the insect cuticle. Whilst Gupta, Leathers, El-Sayed and Ignoffo (1992) showed that strain variability in *B. bassiana* was significant and that the cuticle source of the media on which the fungus was grown affected the expression of the enzyme.

Kmitowa, Bajan and Wojciechowska (1977) noted that isolates of *Beauveria bassiana* tended to be host specific. They suggested that host specificity may be due to variations in cuticle composition from species to species, or to variations in enzyme production, particularly chitinases, by different fungal isolates. However it is also likely that there is a considerable variation in toxic metabolite production between isolates and this would affect host specificity.

Wasti and Hartmann (1975) found that spores trapped in the inter-segmental folds of the cuticle of the gypsy moth, *Porthetria dispar*, were more likely to penetrate the host than if they were on the surface, though they also found that fungal spores could enter through the mouth, germinate in the mid-gut and then penetrate the gut wall and pass into the haemocoel. Clark, Kellen, Fukuda and Lindgren (1968) demonstrated that *Beauveria bassiana* infected mosquito larvae, *Culex pipiens*, through the perispiracular lobes of the siphon. The fungus caused high mortality in the larvae, but it was unclear if this was due to a blockage of the tracheal trunks in the siphon, or to a toxin. However the observation illustrates the versatility of *B. bassiana* in gaining entry to its host.

Additionally *B. bassiana* spores have been shown to enter the insect via the spiracles, germinate and then penetrate the walls of the trachea (Pekrul and Grula, 1979). Although they observed direct entry of the germ tubes into the host cuticle of the corn earworm, Vey and Fargues (1977) reported appressorial formation prior to the penetration of *Leptinotarsa decemlineata* larvae.

After penetration of the cuticle, fungal mycelium grows within the haemocoel. Hyphal body production in the haemolymph may be followed by proliferation of mycelium and larval death, but fungal invasion of the insect tissues varies from isolate to isolate and species to species (Wasti

and Hartmann, 1975).

The insect's fat body is often the first tissue to be invaded and invasion is likely to be preceded by degradation. At the same time there may be changes in the level of haemolymph proteins (Gardner, Sutton and Noblet, 1979). Extensive mycelial growth within the haemocoel prior to death probably causes death by the physical disruption of the host tissues and by host nutrient depletion, whereas limited hyphal growth within the haemocoel prior to death may indicate toxin production by the fungus. Often after insect death hyphae will grow out through the cuticle to the surface and begin to sporulate.

1.4 Toxin production

Bajan and Kmitowa (1969) demonstrated a direct relationship between conidial dose of *Beauveria bassiana* and pathogenicity in the Colorado beetle *Leptinotarsa decemlineata*. The relationship of conidial dose to pathogenicity may be due to toxic metabolite production. The greater the conidial dose the greater the quantity of toxins produced and subsequent insect mortality.

Kucera and Samsinákóvá (1968) showed that *B. bassiana* produced substances *in vitro* which were toxic to *Galleria mellonella* larvae and which were later identified as proteolytic complexes They also showed that the activity of the toxic substances was enhanced by nutrient nitrogen sources. These sources might provide easily transferable amino acids, or possibly critical amounts of trace elements.

When insect death occurs at a time when infection is still confined to the haemolymph, toxin involvement is likely. Kodaira (1961) had earlier shown that haemolymph from *Galleria mellonella* larvae infected with *B. bassiana* was toxic to healthy waxmoth larvae on injection and confirmed that *B. bassiana* could kill these larvae both by mycosis and toxicosis. He showed that toxins might be harmless *per os*, or by contact, and only effective by injection, and hence of little use in insect pest control..

Kodaira (1961) also showed that infection by *B. bassiana* led to the appearance of antibacterial activity in silkworms and that the mummified cadavers were highly antibiotic, thus explaining why they were preserved for long periods without bacterial degradation. This anti-bacterial activity was presumed to be due to fungal metabolites which although not toxic to *Bombyx mori* might be toxic to other insect species. However, it should be noted that it is possible that the antibacterial substance(s) could have been produced by the infected insect rather than the fungus.

El Basyouni, Brewer and Vining (1968) suggested that variations in the morphology

and pigmentation of cultured fungus indicated that *B. bassiana* was metabolically diverse. Roberts and Yendol (1971) also noted this and stressed that a single fungal species could contain widely divergent strains. They suggested that only single-spore, or single-nucleus isolates should be used to limit the risk of variation. To reduce this risk they recommended that stocks of isolates should be stored at low temperatures so that frequent transfer was unnecessary and the chance of mutation reduced.

The use of standardised strains which are shown to be uninucleate by cytological testing is important since isolates recovered from the wild may be heterokaryotic and are more likely to be variable in toxin production and pathogenesis.

Since some fungi produce a range of toxic compounds which may act simultaneously it becomes important to separate the components of a toxic complex. The more a toxin can be purified and defined the better the mechanism of its action can be judged without interference from other biologically active compounds which might contribute to the complete toxic effect (Lysenko and Kucera, 1971).

Some toxic breakdown products may be present when the medium in which the fungus has been grown is examined for toxicity (Kodaira, 1961). Alternatively the toxic products may only be produced in the insect body, which is probably the case with hydrolytic enzymes.

West and Briggs (1968) investigated toxin production by *B. bassiana* isolates *in vitro* by injecting extracts into *Galleria mellonella.* They found that isolates from infected insect hosts were better toxin producers than those repeatedly sub-cultured on media. They were unable to identify the toxin or even to establish whether it was a complex or not, but they did find that the titre varied with the ageing of the culture. This reinforced the idea that the production of metabolites is probably variable in quantity as well as in type, and that the age of the culture affects the production.

Toxins of high molecular weight may be fungal enzymes which could be of considerable interest in insect pathology. These products are often secreted into the culture medium and so can easily be investigated. Enzymes have already been mentioned as attacking the cuticle. When secreted into the host body, they may be able to digest tissues and haemolymph constituents and thus act as toxins. Lipolytic enzymes and glycogenase have been extracted from cultures of *M. anisopliae* (Huber, 1958) whilst *B. bassiana* has been found to produce proteolytic enzymes and amylase. Kucera and Samsinákóvá (1968) separated proteases of *B. bassiana* from compounds of low molecular weight in culture broth by gel filtration. The proteolytic complex which was toxic to *G. mellonella* could be separated into two components and was of moderate toxicity.

Proteases are able to damage very quickly, and specifically, some principal physiological functions by attacking biologically active proteins. St Leger, Joshi, Bidochka and Roberts (1996b) showed that it was possible to manipulate the gene encoding regulation of a cuticle degrading protease, in *Metarhizium anisopliae*. It then became possible to engineer the fungus to overproduce toxic proteases and to increase its pathogenicity towards *Manduca sexta* larvae. It is also interesting to note that the infected insects were rapidly melanised and the cadavers not conducive to fungal sporulation, thereby reducing the persistence of the genetically modified fungus in the environment. Since the technique of manipulating gene regulation has been well defined in *M. anisopliae* it might also be applicable to *B. bassiana,* providing a suitable method of increasing the pathogenicity of this organism.

Although some of the toxins produced by entomopathogenic fungi are undoubtedly lethal, there are instances, for example the destruxins produced by *Metarhizium anisopliae* which initially produce paralysis in *M. sexta* larvae, where the effects dissipate after a few hours. (Samuels, Reynolds and Charnley, 1988). The destruxins produced by *Metarhizium anisopliae* are effective by injection against Lepidoptera. They depolarise the muscle membrane and produce tetanic and then flaccid paralysis, though at low doses the effect is reversible. This suggests that the insect fat body, by action similar to that of the vertebrate liver, is able to detoxify some fungal toxins. Whilst this is true for destruxin, it may be that under conditions where several compounds occur together, the insect's metabolism is unable to detoxify them.

1.5 The insect response

The insect response to fungal attack varies from species to species. However, many insect responses are subtle, and even specific to a fungal isolate.

Some insects, with hardened and waxy exocuticles, do not provide a suitable surface for appressorial attachment and so remain uninfected. The fungus is then not pathogenic since it cannot gain entry into the insect. Vey and Fargues (1977) showed that the larval moult could be an effective defence mechanism, but concluded that it would be most effective when there was only localised hyphal penetration, or the isolates had low enzymatic activity.

Because of their open circulatory system insects need a rapid response to foreign particles, particularly bacteria and fungal spores. The response is carried out primarily by the insect haemocytes which are able to phagocytose and to encapsulate fungal spores. Phagocytic cells are found throughout the animal kingdom serving a nutritive function in the lower phyla and a defensive system in the higher (Roitt, Brostoff and Male, 1989). However, whilst the haemocytes play a major part in defence, Pendland, Hung and Boucias (1993) showed that the surface of the

invading cell was of prime importance in its recognition by the haemocyte. Hyphal bodies of *B. bassiana* produced *in vivo* in *Spodoptera exigua* lacked specific surface residues and were not opsonised, and hence not phagocytosed by the haemocytes. These hyphal bodies continued to circulate in the haemolymph and successfully evade host recognition.

In arthropods, when haemocytes have attacked invading cells, aggregates may be formed. The core of the aggregates becomes melanised and those around foreign objects are coated with glucosaminoglycan-like (GAG) material (Götz, 1986).

Invertebrates lack immunoglobulins, but have a variety of lectins, lytic and anti-microbial factors which may be continuously present in low concentration, as evidenced by the fact that cell-free insect haemolymph reacts with bound cobra venom factor (Roitt *et al.*, 1989).

Both lectins and prophenoloxidase activity systems have been proposed in the arthropod ability to recognise foreign particles (Söderhäll, Cerenius and Johansson, 1994). Lectins have been shown to play an important role in the mediation of cell-based immunity (Pendland, Heath and Boucias, 1988). They interact as cell surface mediators and as opsonins in the plasma. Ligands from bacterial peptidoglycans and fungal cell walls interact with lectins and Unestam and Söderhäll (1977) were able to show that cell wall fragments elicited defence reactions in the crayfish and that the enzyme phenoloxidase becomes converted into its active form in the presence of fungal cell wall β-1,3 glucans. When the pro-phenoloxidase system is activated, phenols are oxidised into quinones and these are in turn converted into melanin.

Chen, Rowley, Newton and Ratcliffe (1999) purified a lectin specific to laminarin, from *Blaberus discoidalis* serum. This lectin may have similar effects to that isolated from *Blaberus craniifer* by Soderhall,Rogener, Newton and Ratcliffe (1988), which enhanced prophenoloxidase and blood coagulation activation. It may also be important in the recognition by the haemocytes of foreign particles.

The system of foreign-particle recognition appears to be very specific. The prophenoloxidase system recognises the difference between foreign microorganisms by their surface polysaccharides which they can bind to (Ochiai and Ashida, 1988). In the case of fungi, ß-1,3 glucans are responsible for eliciting defensive reactions. So a system exists in insects, independent of phagocytes, which is able to react to specific host proteins.

Phenols may provide a measure of resistance to fungal attack and the production of brown, or black pigments is a typical feature of insect response to infection. St Leger, Cooper and Charnley (1988a) found that the production of cuticle-degrading proteases (Pr1 and Pr2) from *Metarhizium anisopliae* was greater on non-pigmented cuticle.

Melanised cuticle may be relatively resistant to enzyme attack, though Pr1 is able to release melanin from the cuticle and does not appear to be affected. Even so the diffusion of fungal enzymes and toxins may be restricted by the melanised sheath which often surrounds the penetrant hyphae and the fungus may be affected by physical limitation.

1.6 Humoral responses of the insect haemolymph to infection

The reactions of insect haemolymph to microbial infection and invasion by parasites have been studied over many years. Development of modern techniques has allowed some of the anti-microbial constituents of the haemolymph produced as a response to infection to be analysed.

The humoral responses of insects vary between species, though generally lysozyme production is increased after infection (Chadwick, 1970). Lysozyme (Chadwick 1970), cecropins (Holak, Engström, Kraulis, Lindberg, Bennich, Jones, Gronenborn and Clore, 1988) and attacins (Hultmark, Engström, Bennich, Kapur and Boman, 1982) and many other metabolites have been shown to be induced by infection.

Lysozymes are basic, stable enzymes able to lyse bacteria by hydrolysing the linkages in the cell-wall peptidoglycan. They are widespread throughout the insect world and play an important role in cell breakdown prior to metamorphosis, however their function in response to fungal attack has attracted little attention.

Hughes, Hurlbert, Rupp and Spence (1983) showed that a range of bactericidal peptides was induced in *Manduca sexta* larvae after bacterial infection.

One of the best studied groups of these inducible anti-bacterial peptides and proteins found in insects is the family of cecropins. These are small peptides which have lytic activity against both Gram-positive and Gram-negative bacteria. They appear to interact with the bacterial membrane and by being firmly anchored to it are held in close contact and are effective in lysing it (Holak, Engström, Kraulis, Lindberg, Bennich, Jones, Gronenborn and Clore, 1988).

Other bactericidal peptides similar to cecropins have been isolated from *Sarcophaga peregrina*, the flesh fly (Okada and Natori, 1983). These peptides, sarcotoxins, were shown to be effective against *Escherischia coli* by disrupting the membrane potential and making the membrane more susceptible to lysis by the detergent sodium dodecyl sulphate (Okada and Natori, 1984).

More groups of anti-bacterial peptides have been identified in Diptera by Keppi, Zachary, Robertson, Hoffman and Hoffman (1986), but appear to be specific and only active against certain bacteria, notably the Gram-negative *E. coli*. However in 1988

Matsuyama and Natori isolated the inducible peptide defensin from *Sarcophaga peregrina.* This was shown not only to be effective against a range of Gram-positive bacteria, but, more interestingly, it was shown to be expressed in larval haemocytes in response to whole insect infection. Often the insect fat body is responsible for the production of induced proteins and peptides, but defensin was induced in haemocytes and it was possible to study its production *in vitro.*

Chernysh, Cocianich, Briand, Hetru and Bulet (1996) showed that after injection with the heat-killed bacteria *E. coli* haemolymph of the hemipteran bug *Palomena prasina* contained four novel peptides. These proline-rich peptides were named metalnikowins and appeared to be bacteriostatic rather than bactericidal. Since the bacteria were dead when they were injected, it was suggested that the insect response was to invasion by bacterial cells rather than to infection.

Lemaitre, Reichhart and Hoffmann (1997) found that *Drosophila,* infected with *B.bassiana,* expressed the gene for the anti-fungal peptide,drosomycin, but that the genes coding for anti-bacterial peptides were not expressed. Braun, Hoffmann and Meister (1998) showed that mutant *Drosophila* larvae (domino larvae), which did not have haemocytes, were still able to synthesze anti-microbial peptides. Humoral reaction to microbial infection stimulated the rapid synthesis, by the fat body, of anti-microbial peptides, which were secreted into the haemolymph. Seven distinct anti-microbial peptides were produced, of which drosomycin was exclusively anti-fungal and metchnikowin both anti-bacterial and anti-fungal. This recent work has extended the knowledge of humoral responses to insect infection and also the way in which anti-microbial peptides are regulated in insects.

Apart from anti-bacterial and anti-fungal peptides, insects can also be induced to produce anti-bacterial proteins. These include a group known as attacins, originally isolated from *Hyalophora cecropia* and *Sarcophaga peregrina.* These proteins act by increasing the permeability of the outer membrane of Gram-negative bacteria and hence the susceptibility of microorganisms like *E. coli* to penicillin (Engström, Carlsson, Engström, Tao and Bennich, 1984). However, even at low concentrations, attacins are effective against a wide variety of other bacteria (Hultmark, Engström, Andersson, Steiner, Bennich and Boman, 1983).

Similar in structure and effect to the attacins is the group of anti-bacterial proteins isolated from *Sarcophaga peregrina* and known as sarcotoxins II, III and IV.

Ando, Okada and Natori (1988) showed that these proteins were effective against actively growing bacteria and, like the attacins, were thought to attack the outer membrane of Gram-negative organisms.

Ferrandon, Jung, Criqui, Lemaitre, Uttenweiler, Michant, Reichart and Hoffman (1998), using drosomycin green flourescent protein reporter gene, showed that *Drosophila* produced an anti-fungal protein in a variety of tissues. They suggested that the insect epithelium was not merely a passive barrier to infection, but an active component of innate immunity.

Undoubtedly further anti-microbial metabolites will be found in insect haemolymph, since the vast number of different insect species are likely to produce a wide range of products in response to microbial infection.

1.7 Cellular responses of larval haemocytes to infection

Whilst the humoral responses of insects to infection are extremely diverse and variable, depending on the insect species and stage of development, most insects which have been studied have remarkably similar cellular responses to infection.

It is tempting to regard cellular responses of invertebrates as down-graded mechanisms of vertebrates whereas they are diverse and have a complexity which is only partially understood. Some cellular responses to infection have similar features across a wide range of insect species. Additionally, some cellular responses found in insects, notably phagocytosis, are similar to those in vertebrates and it is possible that insect cells respond to some of the same stimuli as vertebrate cells.

The responses fall into four recognised categories, co-agulation, phagocytosis, nodule formation and melanised encapsulation.

Co-agulation

If the insect epidermis is damaged a blood clot forms beneath the wound. Formation of the clot involves components from both the haemocytes and the plasma. Granular cells release material which forms a gel. This gel is stabilized by plasma lipoproteins and phenoloxidases from the haemocytes. After clotting has occurred plasmatocytes migrate to the site and haemocytes become bound together to form a continous tissue (Chapman R.F. 1998).

Phagocytosis

Phagocytosis is a common cellular defence response to invasion in both vertebrates and invertebrates. A sequence of events occurs involving interactions between haemocytes and the

invading organisms. If any part of this sequence is disrupted phagocytosis may be prevented.

Haemocytes must first recognise non-self in order to initiate a response. Weisner and Gotz (1993) showed that larval granular cells of the moth *Galleria mellonella* which came into contact with hydrophilic silica beads recognised inert non-self and released a sticky granular material. They found that the surface condition of the beads was important for successful phagocytosis and that hydrophobic beads were not attacked. Both *in vivo* and *in vitro* de-granulated granular cells (GR's) appeared to opsonize the beads which were then engulfed by plasmatocytes (PL's).

Ratcliffe (1985), in his review, points out that there is a large range in the phagocytic activity of different insect species. He stresses the importance of standardising culture conditions, since arthropod blood cells are diverse and only a small percentage are phagocytic.

Horohov and Dunn (1982) showed that *Manduca sexta* larvae were unusually resistant to the bacteria *Pseudomonas aeruginosa* (Strain P11-1) and *Escherichia coli* (Strain D31) but that even so an increase in the larval GR's occurred after bacterial injection. This coincided with a rapid decrease in bacterial numbers and suggested that early phagocytosis might explain the resistance of this insect to bacterial pathogens.

The ability of insect haemocytes to move up a chemical gradient (chemotaxis) as an early phase of phagocytosis has proved difficult to study *in vitro.* Insect plasma gels quickly, even so, Ratcliffe and Rowley (1978) noted movement of plasmatocytes towards bacteria/granular cell aggregates.

In 1991 Anggraeni and Ratcliffe showed the importance of granular cells in the phagocytic sequence. *Galleria mellonella* plasmatocytes, after Percoll separation, were unable to phagocytose *Bacillus cereus* unless they were pre-washed. The addition of GR's to the unwashed PL's recovered their phagocytic ability, suggesting that GR's produce opsonising factors.

Plasmatocytes play a major role in phagocytosis. After the invading organism has become attached to the plasmatocyte the cell membrane may simply invaginate and enclose it. Alternatively, pseudopodia may surround the foreign particle and engulf it in a phagocytic vacuole where a suite of enzymes may start digestion.

Dose-dependency has been considered as a factor in the efficiency of phagocytosis. However Ratcliffe and Walters (1983) showed that although phagocytosis of *B. cereus* and *E. coli* by *G. mellonella* larval haemocytes was dose-dependent at lower doses, at higher doses some strains were cleared more efficiently. This suggested that other mechanisms, like nodulation could be occurring.

Whilst the phagocytosis of bacteria has been well researched less is known of the phagocytosis of fungal cells. Hung and Boucias (1992) suggested that phagocytosed cells of *Beauveria bassiana* were able to produce metabolites which suppressed the phagocytic response of circulating haemocytes of *Spodoptera exigua.* Subsequently Hung, Boucias and Vey (1993) showed that a *B. bassiana* isolate (UF1 5477) could affect the phagocytic competence of *S. exigua* haemocytes. When fungal blastospores were co-injected with the yeast *Candida albicans* the yeast cells failed to elicit a haemocytic response. It was suggested that *B. bassiana* growing *in vivo* might produce immuno-suppressive factors which prevented the recognition of the yeast by the haemocytes.

In 1973 Anderson, Homes and Good found that *Blaberus craniifer* haemocytes were able to kill trapped bacteria within an hour. By contrast, Hung *et al* (1993) showed that the phagocytosed fungal blastospores of *B. bassiana* produced germ tubes and grew out of the engulfing granular cells.

Nodule formation

One of the major cellular responses to invasion, whether by bacteria, fungi, parasitoid eggs or inert substances, is to surround the invading material with layers of cells in a particular sequence and thus to form nodules or capsules.

Gotz (1986) considered that nodule formation by haemocytes occurred against small, particulate material, whilst capsule formation was the haemocytic response to larger invading objects. However the sequence of haemocyte attachment was essentially the same in both nodule and capsule formation.

Ratcliffe and Gagen (1976) described the formation of haemocytic nodules from *Galleria mellonella* larvae injected with killed *Bacillus cereus.* As the circulating haemocytes came into contact with the foreign particles the granular cells (GR's) appeared to attach to them. It is uncertain whether the granular cells encountered the foreign particles by chance, or whether they were able to move up a chemical gradient by chemotaxis. However they were able to recognise non-self, probably due to cell-bound receptors and then to adhere to the foreign surface.

As the GR's attached to the foreign cells they discharged some of the contents of their vacuoles and this sticky material may have been a factor in attracting plasmatocytes (PL's). Small nodules formed surrounding the foreign material and gradually increased in size as more haemocytes of both types became attached. Eventually some of the outer GR's completely disintegrated, leaving a nodule composed of flattened plasmatocytes and granular cells surrounding the foreign material.

In 1977 Ratcliffe and Gagen further considered the role of granular cells in nodule formation and suggested that the granular discharge was unusual and that the cell membrane of these cells might be highly sensitive to non-self and break down in the presence of foreign bodies.

Although a number of different workers (Dunn and Drake,1983 ; Hung and Boucias, 1992 ; Weisner and Gotz, 1993) have investigated phagocytosis by insect haemocytes, Ratcliffe and Walters (1983) considered both phagocytosis and nodule formation together.

By injecting *Galleria mellonella* with strains of *Bacillus cereus* and *Escherichia coli* they showed that the larval haemocyte count was related to the dose and pathogenicity of the bacteria. The more highly pathogenic organisms produced a greater nodular response which, in the case of *B. cereus*, was dose-dependent. There was a positive correlation between the speed with which nodules were formed and the pathogenicity of the micro-organisms injected. However *B. cereus* was able to escape from the haemocytic nodules and elicit an increased host response, showing that entrapment by haemocytes was not always lethal to invading organisms.

Pech and Strand (1996) examined the entrapment and encapsulation of Dowex 1x-2 beads by *Pseudoplusia includens* haemocytes *in vitro*. They showed that the beads were surrounded by haemocytes in a particular sequence. The first cells to attach to the beads were granular cells and these were then followed by over-lapping layers of plasmatocytes. After this a monolayer of granular cells adhered to the outer layer of plasmatocytes and further nodule formation ceased. The same pattern occurred *in vivo* as *in vitro* after the Dowex beads had been injected into *P. includens* larvae.

However, when gradient-purified granular cells were incubated with the beads, although a few of the haemocytes attached, they did not form over-lapping layers. Similarly, highly purified plasmatocytes were unable to form nodules around the beads unless they were pre-incubated with granular cell-conditioned media. Pech and Strand (1996) considered that they had clearly demonstrated a cell-to-cell signalling which affected nodule formation around inert material.

Although the nodule formation by haemocytes in response to infection has been demonstrated by many workers, in 1994 Kanost, Zepp, Ladendorff and Andersson uncovered further complexities in the mechanism of haemocyte aggregation.

They showed that a protein in *M. sexta* haemolymph, which they called haemocyte aggregation inhibitor protein (HAIP), was able to prevent haemocyte aggregation. This 50kDa protein was constitutively expressed and not induced by bacterial injection. Since haemocyte aggregation is a major cellular response to infection, the role of a protein that abrogates this response is puzzling. HAIP from larval haemolymph had been purified and considerably

concentrated before the aggregation work on haemocytes had been carried out. Possibly at normal levels its effects on cell aggregation were unmarked and it fulfilled a different function.

Encapsulation

Encapsulation follows nodule formation and involves the melanisation of multi-layered cellular envelopes around foreign objects in the insect haemocoel, thus restricting the growth, or movement, of the invader.

As with nodule formation, encapsulation starts with granular cells (GR's) reacting to non-self, de-granulating and adhering to the foreign body. Plasmatocytes (PL's) are then attracted and eventually both types of haemocytes form capsules of 5 to 30 tightly packed layers of flattened cells around the foreign object. Melanin deposits form in the inner layers of the capsule which may then become completely melanised and mummify the invading object. Eventually the size of the capsule reduces the attracting signal from the activated GR's, preventing further growth and allowing peripheral cells to disperse.

The encapsulation of parasitoids by insect haemocytes is a well-documented occurrence (Ratner and Vinson, 1983 ; Stoltz, Krell, Summers and Vinson, 1984 ;Strand and Noda, 1991 ; Harwood and Beckage, 1994 ; Russo, Dupas, Frey, Carton and Brehelin, 1996) and demonstrates the complexity of host / parasite interactions. Those studied give an insight into the mechanism of haemocyte aggregation and the way in which this reaction may be suppressed.

Stoltz *et al* (1984) reported that polydnaviruses (PDV's) which had replicated in the calyx cells of the ovaries of female wasps and were injected with the eggs of the parasite into the host haemocoel caused immuno-suppression and the inability to encapsulate the parasite. It seemed possible that these viruses either carried a substance on their surfaces, or induced a substance in the haemolymph which suppressed the normal capsule-forming response of the insect haemocytes.

Ten years later Harwood and Beckage (1994) compared the injection of *Manduca sexta* larvae with purified PDV's in non-parasitized insects with those parasitized by *Cotesia congregata*. They showed that the purified PDV induced the production of " early proteins" in the insect and speculated that these might inhibit immuno-recognition by the host in the same way that lectins, as surface active molecules, modulate the immune function .

The molecular mechanism by which PDV's are able to disrupt the encapsulation of braconid eggs by the haemocytes of *Pieris rapae* was demonstrated in 1997 by Asgari, Schmidt and Theopold. PDV-encoded protein prevented the cell surface exposure of lectin binding sites. Since the exposure of lectin-binding cell surface glycoproteins and the formation of microparticles are a prerequisite of cell-mediated encapsulation, a virus-encoded protein which can inactivate this

suppresses the immune response of the parasitized host.

The wasp, *Cotesia rubecula*, carries a polydnavirus which infects the host haemocytes and fat body cells. The cells then secrete this into the haemolymph where it interacts with the surface of the haemocytes and precludes normal cellular encapsulation. It appears that PDV effects on haemocytes are due to a single glycoprotein.

Successful parasitism requires that the parasite evades the hosts immune responses such as encapsulation and certainly PDV's appear to abrogate the host immunity.

1.8 The role of prostaglandins in haemocyte aggregation

Undoubtedly the cell membrane plays a major role in the recognition of non-self and where an agent affects the membrane surface the immune response will be affected.

Nodule formation by larval haemocytes may be influenced by the presence in the haemolymph or on the cell membrane of active substances like the prostaglandins. Prostaglandins are C20 carboxylic acids with a cyclopentane ring. In mammals they have been shown to have a wide range of functions including the maintenance of body temperature, blood pressure and the protection of organs from damage caused by trauma and disease. They are also implicated in arthritis, malignancy, allergic reactions and the immune response. Arachidonic acid is the most important prostaglandin precursor and is formed when cell membrane diacylglycerides are broken down. It is then metabolized to form a series of products including a range of prostaglandins and leukotrienes (See diagram below)

Inaccessible phospholipids on cell membrane surface

↓ ←stimulus causing disruption

accessible phospholipids

↓ ←phospholipidase

arachidonic acid

lipooxygenase ↓ ↓ cyclooxygenase

leukotrienes prostaglandins and thromboxanes

(C20 compounds)

Prostaglandins (PG's) have important effects on the vertebrate immune system. Bonta and Parnham (1982) suggested that mammalian lymphocytes were responsive to PG's and sensitised macrophage cells during chronic inflammation. PG's are probably also important regulators of the invertebrate immune system. In 1992 Hampson, Rowley, Barrow and Steadman showed that the prostaglandin precursor, arachidonic acid was abundant in the haemocytes of the

shore crab, *Carcinus maenus.* The blood cells were able to synthesise PG's but the synthesis was inhibited in a dose-dependent manner by the cyclooxygenase inhibitor, indomethacin.

A review by Stanley-Samuelson (1994) discussed the significance of prostaglandins in insect physiology and emphasized their diversity of roles. It was suggested that PG's regulate events within the insect tissues and cells and mediate the cellular immune response. Although these biologically active molecules are well-known, they have proved difficult to study because of their ability to change from one form to another and also because of their multiple actions on cells.

In 1991 Stanley-Samuelson showed that prostaglandins could reverse the inhibition of the cellular immune response of *M. sexta* larvae treated with dexamethasone. Later, Miller, Nguyen and Stanley-Samuelson (1994) showed that inhibition of eicosanoid biosynthesis by dexamethasone reduced nodule formation within an hour of infection of *M. sexta* by the pathogenic bacteria *Serratia marcescens* and that this inhibition could then be reversed with the eicosanoid precursor, arachidonic acid.

Subsequently Stanley-Samuelson and Ogg (1994) showed that although *M. sexta* fat body was able to produce prostaglandins it was unable to inter-convert them and synthesis was inhibited by indomethacin at low doses. Miller, Howard, Nguyen Rosario and Stanley-Samuelson (1996) found similar results with the tenebrionid beetle, *Zophobas atratus* showing that dexamethasone could inhibit the nodule-forming response and arachidonic acid could rescue the reaction in different species of insects. The same group, in 1997, used the silkworm, *Bombyx mori*, to show that arachidonic acid could also rescue the nodule-forming response of larvae treated with the inhibitor dexamethasone. This extended the number of insect species whose haemocytes were known to react in this way although as Stanley-Samuelson pointed out, there exists tremendous variation in insect physiology and it may be unsound to generalise from one species to another.

In 1999, Miller, Howard, Rana, Tunaz and Stanley added another insect species, the cricket, *Gryllus assimilis*, to the list of those whose haemocytic nodule formation was inhibited by dexamethasone and rescued by the injection of arachidonic acid.

This large group of similar reactions from different insect species showed that although the immuno-suppressive mechanism might vary between species, inhibition by dexamethasone and recovery with arachidonic acid was not unusual. Most of this work was carried out *in vivo* and subsequent work *in vitro* might help to clarify the effects of eicosanoids on the cell aggregation without the presence of other components of the haemolymph.

Mandato, Diehl-Jones, Moore and Downer (1997) working with *Galleria mellonella*

both *in vivo* and *in vitro* showed that both dexamethasone (a phospholipase inhibitor) and indomethacin (a cyclooxygenase inhibitor) reduced phagocytosis and nodule formation. These reactions could be reversed *in vivo* and *in vitro* by the injection or addition of arachidonic acid, suggesting that the same agents were able to affect both phagocytosis and nodule formation.

In the light of a considerable body of work on the effect of eicosanoids on larval haemocyte aggregation it is important to determine if the metabolites of *Beauveria bassiana* work in a similar way to inhibit nodulation and if so whether their effects can be reversed by arachidonic acid.

The mechanisms by which some invading organisms are able to reduce the ability of haemocytes to phagocytose remain to be studied. The way in which insect haemocytes form nodules to combat infection and the fungal response to this reaction is complex and deserves further study.

1.9 Aims and rationale for the present work

The aim of the work was to distinguish between twelve different *Beauveria bassiana* isolates on the basis of their pathogenicity and toxicity to *Manduca sexta* larvae.

Although all the isolates used were of lepidopteran origin, none had been isolated from *M. sexta*, and since *B. bassiana* isolates vary a great deal in their pathogenicity to different insect species it was possible that some would not be pathogenic to this species.

An important innovation in this work was to identify which isolates would be able to penetrate the insect cuticle and which would only be effective by injection. To this end all twelve isolates were tested on *M. sexta* larvae using two different types of treatment, immersion in spore suspension, or injection, of conidiospores through the inter-segmental membrane. Previous work on *B. bassiana* had tested pathogenicity only by sprays or dip tests.

Fungal isolates able to gain entry to the insect through the cuticle need to produce chitinolytic and proteolytic enzymes, probably after appressorial formation, whilst those able to develop only after injection through the cuticle might possess an inadequate suite of enzymes or respond to a different range of elicitors.

Nevertheless, such isolates might still possess attributes sufficient to cause disease once past the cuticle barrier. An important component of such attributes is the ability to produce toxins. Some indication of this can be gained from dose-response relations revealed by probit analysis.

It is quite possible that *B. bassiana* produces metabolites which, although toxic to some insects, do not affect *M. sexta* and inevitably would pass unnoticed. Additionally the fungus may

produce low levels of toxic products which do not cause insect death. However, careful monitoring of insect weight gains can indicate the effect of toxins which might not be apparent from lethality studies alone.

Symptomology and the progress of disease are also useful indicators of toxin involvement. To this end experimental protocols were devised that allowed recording of symptoms, and sampling of haemolymph which could be examined for evidence of mycosis.

Bioassays, using the filtrates from *in vitro* cultivation of the different isolates might show the presence of toxic metabolites produced by mycelia. Trials like these were used to distinguish between isolates producing high and low amounts of toxic metabolites. Particular attention was paid to the presence of different metabolites including fungal pigments like oosporein and salts, like calcium oxalate.

Dose-response trials were used to highlight the differences between isolates, and showed which were likely to affect larvae by mycosis and which by toxicosis. Although trials of unanalysed filtrate cannot identify toxins, their effects on insects could be used to indicate the type of metabolites present and such effects as pigmentation and paralysis could be used as indicators.

A study of the larval haemolymph was undertaken to help to clarify the complex interaction between fungal spore, filtrate and larval haemocytes. Changes in the number of the various cell types were followed, and interpreted according to these principles. The action of fungal filtrate from the growth of different isolates of *Beauveria bassiana in vitro* on the aggregation of larval haemocytes, was compared.

The diverse mechanisms which different isolates possess allow some to be effective pathogens whilst others remain completely ineffective. This work aimed to identify some of these factors which differentiate between pathogenic and non-pathogenic isolates of *B. bassiana.*

Chapter 2

The pathogenicity of *Beauveria bassiana* isolates toward *Manduca sexta* larvae.

2.1 Introduction

Beauveria bassiana has been recognised as an insect pathogen for over 150 years. It has a wide range of insect hosts and it is probable that different isolates of this ubiquitous fungus employ different strategies in the destruction of their insect hosts.

The twelve isolates studied all originated from lepidoptera, though none from the target insect, *Manduca sexta*.

The first objective was to determine the ability of the different isolates to penetrate the larval cuticle. Successful colonisation of the host is not dependent solely on factors intrinsic to the host-parasite relationship. To be a successful pathogen, the fungal spores must be able to penetrate the insect cuticle and the fungus must be able to develop within the insect under the conditions in which the host lives. Some fungal species whilst possessing the ability to penetrate the host cuticle are not successful insect pathogens. They may require high levels of humidity, or be unable to withstand high ambient temperatures which would normally occur in the insect's habitat. These are factors which must be taken into account in assessing the utility of a particular fungal isolate for practical insect control (Bidochka, Miranpuri and Khachatourians, 1993).

A comparison of the pathogenicity of the spores of different *B. bassiana* isolates with two distinct methods of infection (immersion and injection) might show which were able to breach the cuticle and which were only able to grow if injected into the haemolymph. Additionally, by measuring the daily weight gain of treated larvae, it should be possible to show which fungal isolates affect the growth of the insects but are unable to kill them.

Under normal conditions fifth instar larvae will take 5 or 6 days between their last moult and the time at which they stop feeding and prepare to pupate (wandering stage). They do not normally reach this stage at less than 8 grams live weight. If fungal treatment reduces the rate at which larvae gain weight this may provide a good indicator of fungal pathogenicity. Similarly, if after treatment with conidiospores the amount of faecal pellets produced is reduced this might also indicate a response to fungal infection, albeit at a sub-lethal level.

2.1.1 Conidial concentrations

Conidial concentrations used to treat the larvae may be critical. With immersion treatment low concentrations of spores on the surface of the insect could even allow sufficient spores to be removed by self-grooming to give erratic or negative results.

The concentration of spores injected into the haemocoel might also be an important factor in pathogenicity. Low concentrations of spores might be rapidly phagocytosed or surrounded by haemocytes. The insect response to low fungal doses might mask the actual capability of an isolate as a pathogen.

The use of *Beauveria bassiana* spores for insect control has been successful for a wide range of species, but the mechanism by which the fungus destroys its host is only known for some species and some isolates. It is likely that a fungus with such a wide geographical distribution and large number of insect hosts will have more than one way of attacking its host and that isolates may well produce different toxic metabolites.

Injection of spore suspensions may highlight those isolates which, although unable to breach the insect cuticle, are able to produce toxic metabolites once they are inside the host. Examination of the haemolymph after injection of spore suspensions may show if the fungus is able to grow within the insect, whether it has an effect on the insect haemocytes and whether it can cause death by mycosis.

By comparing two different methods of treatment for each isolate not only pathogenicity, but varying fungal strategies may become apparent. If isolates are pathogenic after injection, but not when insects are immersed in spore suspension, it is possible that the fungus is unable to produce the necessary enzymes to penetrate the insect cuticle. Inoculation of excised cuticles with conidiospores could determine whether the fungus was able to penetrate the cuticle, yet unable to develop subsequently.

An examination of haemolymph after injection of spore suspensions will show if the fungus, whilst unable to breach the cuticle, is able to grow within the insect and possibly cause death by mycosis.

2.2 Materials and methods

2.2.1 Fungal culture.

Twelve isolates of *Beauveria bassiana* were obtained from the USDA collection held at The Boyce Thompson Institute for Plant Research, NY, USA (See Appendix). They were chosen because they had originally been collected from Lepidoptera.

Spores were harvested from the original cultures in 0.05% Tween 80 and stored in sterile 10 % glycerol at -20° C. Stock plates were made on Sabouraud's Dextrose Agar with 2% added yeast extract (SDAY) from the stored spores after the latter had been allowed to thaw for 1 hour at 25° C. These stock plates were stored at 4° C and used to inoculate SDAY plates. Stock plates were renewed every 4 months from stored spores. Inoculated plates were incubated at 25° C ± 1° C for 14 days in the dark and sporulation was confirmed prior to harvesting.

Spores were harvested using sterile 0.05 % Tween 80. There was little difference in yield of spores harvested in distilled water, or 0.05 % Tween 80. After harvesting the spores were centrifuged at 1407*g* for 15 minutes, the supernatant decanted and the residual spores washed a further three times with sterile distilled water. The spores of each isolate were then suspended in sterile distilled water and shaken on a vortex mixer for one minute to ensure that any clumps were broken up. Each suspension was then diluted to one tenth, or one hundredth in distilled water and counted using a Neubauer haemocytometer.

2.2.2 Experimental insects stock culture

Freshly ecdysed fifth instar *Manduca sexta* larvae were placed in individual plastic pots with a suitable quantity of Manduca diet (see Appendix). They were handled by the horn to avoid damage.

The individual containers were placed on damp absorbent paper in plastic tubs with lids to maintain a high humidity. Incubation was at 25° C with a 17 h / 7 h light/dark regime and an incubator humidity of 45%.

Insects which had stopped feeding and were seeking a pupation site (wandering larvae) were placed in clean individual pots with absorbent paper and pierced lids to pupate. Pots containing wanderers were placed on their sides and incubated at 25° C, with a 17 h / 7 h light/dark regime.

2.2.3 Injection and immersion using spore suspensions

Suspensions of twelve isolates were made in sterile distilled water. The number of spores was counted with a Neubauer haemocytometer and four dilutions made between 1×10^4 and 5×10^7 spores per ml.

Freshly moulted fifth instar *M. sexta* larvae were weighed and either injected with 10 µl of the appropriate dose of spores into the inter-segmental membrane between the 7^{th} and 8^{th} segment, slightly to one side of the mid-line, or immersed in the appropriate spore suspension for 10 seconds. Larvae were then replaced in individual pots which were put on dampened absorbent paper to maintain high humidity. The insects were fed normal *Manduca* diet and incubated at 25° C ± 1° C, with a 17 h / 7 h light/dark schedule. They were inspected daily, scored as dead or alive, recorded for melanisation and weighed.

This assay was repeated twice for all 12 isolates, each with 5 insects at four different concentrations. Controls were injected with 10µl of sterile distilled water or immersed in sterile distilled water.

2.2.4 Calculation of LC_{50}

LC_{50} is defined as the concentration which is lethal to 50% of the animals tested.

The LC_{50} for all the isolates which were injected was calculated for results at 72 and 96 hours, using the method defined by Finney (1964). This was less appropriate

for larvae treated by immersion, since only the highest concentrations were effective. Dosage-mortality data for injected insects was probit transformed in order to determine the median lethal concentrations (LC_{50}) for each isolate.

2.2.5 Comparison of faecal weights from immersed larvae.

Dried faeces can be used as an indication of an insect's ability to digest its food and to develop normally. The faeces from each group of 5 larvae, which had been immersed in 4 different spore concentrations for each isolate, were collected every 24 hours. The faeces were put in a dried and pre-weighed aluminium boat. They were then dried at 65° C to a constant weight. The work was replicated three times.

2.2.6 Ability of different fungal isolates to penetrate the larval cuticle

A variation was evident between the ability of different isolates to kill *M. sexta* larvae after immersion in spore suspensions. The possibility that some isolates were unable to penetrate the cuticle was tested.

The isolates 959, 1122 and 1629 which were unable to cause larval mortality after immersion were compared with isolates 304 and 2727 which caused more than 65% larval mortality at 7 days.

Cuticle ghosts were prepared from freshly moulted 5th instar larvae. Larvae were placed on ice for 30 minutes to desensitise them. The head was removed with flamed scissors and the insect opened ventrally from head to tail. The internal contents and muscle were removed by scraping with a scalpel. This stage was completed rapidly to limit cuticle melanisation. When the muscle had been removed and the cuticle had become transparent it was placed in a stirred saturated solution of phenylthiourea (PTU) for 1 hour to prevent melanisation. It was then transferred to a bath of 5% sodium hypochlorite solution for sterilisation and rinsed in four changes of sterile distilled water.

Using sterile techniques, the cuticles were laid on plates of Sabauraud Dextrose Agar with 2% added yeast extract (SDAY) and allowed to dry for 10 minutes in a laminar flow cabinet. Then 50 µl of spore suspensions of 5×10^6 spores per ml in distilled water of the five different isolates was applied to the surface of the cuticles.

After inoculation, the plates with the cuticles were incubated at 25° C without light from 24 to 96 hours. The cuticles were then transferred to fresh SDAY plates and

both sets of plates were re-incubated.

The addition of the antibiotics, cyclohexamide and chloramphenicol, to SDAY was found to be unnecessary since sterilisation of the cuticle with hypochlorite prevented bacterial contamination.

Ten microlitre samples of spores were removed from each cuticle after 24 hours and the spores counted and scored for germination using an Olympus microscope and phase contrast (× 400). The adherence of appressoria to the cuticle made the ratio of germinated to non-germinated spores unreliable after longer incubation.

2.2.7 Examination of haemolymph

Dunn and Drake (1983) determined the volume of haemolymph of 2d5L *Manduca sexta* larvae using ^{14}C inulin and found a mean of 1.332ml per insect. In the current work the volume of larval haemolymph was determined by injecting the dye amaranth. First day fifth instar larvae had approx. 100µl per insect,rising to 630µl at five days.

Five fifth instar larvae were injected with 10 µl of 1 x 10^6 per ml spore suspension (1 × 10^4 spores) for each isolate. The insects were placed in individual pots with appropriate diet and incubated at 25° C ± 1° C, 17 h/ 7 h light/dark and 45 % RH..

An insect from each isolate treatment was removed at 24 hour intervals. The insect pot was placed on an ice-bed for 10 min. The larva was held with forceps by the horn and washed with distilled water and dipped in 1 % bleach for 30 seconds to destroy external fungal spores, rinsed and then dipped in 70% ethanol for 10 seconds. It was then rinsed with distilled water and the horn was removed with flamed scissors. The haemolymph was collected in sterile 1.5 ml microcentrifuge tubes containing several crystals of phenylthiourea to inhibit melanisation.

The haemolymph was examined and blastospores, hyphal bodies and haemocytes were scored using a Neubauer haemocytometer and an Olympus microscope at × 400 with phase contrast.

2.2.8 Changes in appearance of *M. sexta* larvae.

A record was kept of differences in appearance, melanisation and pigmentation of treated and control insects.

2.2.9 Use of lectins to label fungal elements in the haemolymph

Two different lectins were used with the haemolymph to show whether fungal spores or mycelial fragments were present; Calcofluor which binds to ß-glucan cell wall and Fluorescein isothiocyanate conjugated with wheat germ agglutinin (FITC-WGA) which is specific for oligosaccharides of N-acetyl glucosamine and binds to chitin.

The Calcofluor solution was made up with 50 µg per ml in Tris HCl buffer at pH 0.8 and stored in the dark at 4° C. The FITC-WGA was made up with 200 µg in 0.4 ml phosphate-buffered saline (0.02 M potassium phosphate buffer containing 0.8% sodium chloride) at pH 7.4 and was also stored in the dark at 4° C.

Haemolymph samples were divided into two aliquots in microcentrifuge tubes. 50 µl of FITC-WGA was added to one sample and 50 µl of Calcofluor to the other. Both samples were mixed on a vortex mixer for 1 min at 22° C and then samples were placed on glass slides with cover slips and observed with an Olympus fluorescent microscope using an excitation filter at 490 nm and a barrier filter at 535 nm. The pattern of fluorescence of fungal elements was observed. Although lectin-labelled fungal fragments fluoresced under UV light, the inability to see haemocytes, or to use a haemocytometer to quantify results, meant that this was a limited method.

Haemolymph samples were also observed with a light microscope. The blastospores, hyphal fragments and haemocytes were counted using a haemocytometer at 24 hour intervals after inoculation. All haemolymph assays were repeated twice.

2.3 Results

2.3.1 Mortality of fifth instar *M. sexta* larvae treated by immersion and injection with spore suspensions of 12 different isolates of *B. bassiana*

Immersion in spore suspensions gave consistent results from all three replicates (Table 1). Of the twelve isolates tested only eight were able to kill the larvae after immersion. Two of these eight isolates, 304 and 2727, produced more than 60% mortality at six days, whilst the remaining six isolates produced less than 50% mortality at six days.

Every isolate tested was pathogenic by injection. With the exception of isolate 1558, the LT_{50} varied between 72 and 96 hours with isolate 304 producing 33% mortality after 48 hours, but isolate 1558 only producing 33% mortality after six days. Eight of the isolates caused 100% mortality six days after injection, 3527 doing this by 96 hours. There were no deaths among the control insects for any of the immersion or injection assays.

There were considerable differences between the isolates in their speed of kill. This variation may be associated with their ability to grow within the insect and either their production of invasive mycelia or toxic metabolites. Speed of kill might also be attributed to the concentration of the spore suspension. In some isolates (1122, 2417) LT_{50} at injections of 1×10^5 spores per ml (1×10^3 spores) appeared to lag 24 hours behind injections of 1×10^6 spores per ml (1×10^4 spores). At 6 days four of the twelve isolates produced 100% mortality at both spore concentrations, though overall there was a 91% mortality at 6 days with 1×10^6 spores per ml concentrations (1×10^4 spores), as opposed to 81% mortality with 1×10^5 spores per ml (1×10^3 spores).

Table 1 Percentage mortality of *M. sexta* larvae 7 days after immersion in spore suspension

Isolate No.	Concentration per ml	% mortality
304	4×10^6	73
959	4×10^6	0
1007	4×10^6	20
1122	1×10^7	0
1315	2×10^6	0
1484	2×10^6	33
1558	1.8×10^6	7
1629	5×10^7	0
1886	4.5×10^7	33
2417	9×10^6	7
2727	9.5×10^6	67
3527	1×10^7	40

(5 insects for each isolate replicated 3 times)

Five first day fifth instar larvae were immersed for 10 seconds in spore suspensions prior to incubation at 25°C for 7 days. Of the twelve *B. bassiana* isolates tested by immersing the insects in spore suspension (Table 1) 304 and 2727 were the most pathogenic. Over three replicate tests with five insects in each test the pathogenicity at seven days varied little, but insects which had not died by 10 days went on to pupate and then emerge normally.

Table 2 (overleaf) shows the percentage mortality of larvae after injection with different concentrations of *B. bassiana* spores.

Table 2 Percentage mortality of *M. sexta* larvae after injection of 10µl of different concentrations of conidiospores

Isolate No.	Conc. per ml	48 Hours	72 Hours	96 Hours	7 Days
304	1×10^6	33	73	93	100
	1×10^5	0	60	93	100
959	1×10^6	0	20	66	87
	1×10^5	0	0	0	27
1007	1×10^6	0	75	75	95
	1×10^5	0	25	50	75
1122	1×10^6	0	40	87	93
	1×10^5	0	0	73	100
1315	1×10^6	0	40	87	93
	1×10^5	0	0	73	100
1484	1×10^6	0	53	93	100
	1×10^5	0	0	100	100
1558	1×10^6	0	6	33	33
	1×10^5	0	0	6	33
1629	1×10^6	0	27	87	100
	1×10^5	0	0	60	93
1886	1×10^6	0	67	93	100
	1×10^5	0	0	40	93
2417	1×10^6	0	47	80	87
	1×10^5	0	6	53	67
2727	1×10^6	13	60	87	100
	1×10^5	0	40	87	100
3527	1×10^6	13	67	93	100
	1×10^5	0	67	100	100

Five insects were injected with 10µl of spore suspension (2 different doses) of each isolate. The work was replicated 3 times (n = 15). Larvae were then incubated at 25°C for 7 days and the mortality recorded daily.

Table 3 shows the LT_{50} for larvae injected with the same conidial dose of different *Beauveria bassiana* isolates.

Table 3 LT_{50} for *M. sexta* larvae after spore injection.

10 µl of 1×10^6 conidia per ml

Isolate	LT_{50}
304	58 h
959	87 h
1007	64 h
1122	77 h
1315	78 h
1484	68 h
1558	>168 h
1629	81 h
1886	66 h
2417	75 h
2727	67 h
3527	64 h

n = 15

Five first day fifth instar larvae were injected with 10µl of each of the different isolate spore suspensions and incubated at 25°C for up to 7 days. The work was repeated three times. Calculated LT_{50} values after injection are shown in Table 3. Calculation of LT_{50} values for larvae after immersion was inappropriate since in only two out of twelve isolates was mortality greater than 50% at 7 days. However a comparison between the percentage mortality of larvae after injection (Table 2) and after immersion (Table 1) indicates differences in the fungal strategy of different isolates.

The five most effective isolates after immersion (304, 1484, 1886, 2727, 3527) were more effective pathogens after injection. Even isolates which were totally ineffective by immersion (959,1122,1315,1629) were able to cause considerable mortality after injection.

Isolates which were effective by immersion (304,1484, etc) were quicker to cause mortality by injection.

2.3.2 Effect of concentration of spores on larval mortality (LC_{50})

Table 4 (overleaf) shows median lethal concentration of conidia at 72 h after larval injection, whilst Table 5 gives LC_{50} values for isolates at 96hr together with statistical details of the probit lines.

With two isolates, 1315 and 1886, the data at 72 hours was unsuitable for probit analysis, though suitable at 96 hours. Insects which had been immersed had mortalities which were too low to evaluate by probit at 96 hours.

Beauveria bassiana isolates differed in their virulence, as reflected by the length of time taken to kill larvae when different concentrations of spores were injected. The differences between isolates in LC_{50} at 72 and 96 hours may reflect a variety of fungal strategies.

Gupta, Leathers, El-Sayed and Ignoffo (1994) suggest that fungal pathogenesis is a complex and multifactorial phenomenon with particular virulence factors coming into play at different stages of infection and it seems that this could be the case with the *Beauveria bassiana* isolates tested.

Meynell and Meynell (1965) suggest that an LC_{50} probit line slope of more than two indicates that a pathogenic organism produces a toxin. At 96 hours four isolates 1007, 1122, 1886 and 2727 had slopes of more than two and these isolates may cause larval death by toxicity rather than mycosis.

Five isolates 304, 1315, 1484, 2417 and 3527 had slopes of less than 1 at 96 hours after treatment. It is suggested that these isolates may be effective by causing a reduced feed intake, malaise and finally mycosis, or that they may produce toxic metabolites later than the other isolates.

Table 4 Concentration of *Beauveria bassiana* conidia from different isolates which caused 50% mortality of *M. sexta* larvae (LC_{50}) at 72 hours after injection

Isolate No. ↓	72 Hours post injection	
	LC_{50}	95% confidence limits
304	1.2×10^4	2.0×10^5 1.9×10^3
1007	9.2×10^5	1.0×10^6 6.1×10^5
1122	2.8×10^6	4.8×10^6 1.0×10^6
1315	N/A	N/A
1484	1.7×10^6	2.9×10^6 8.5×10^5
1886	N/A	N/A
2417	1.0×10^8	2.1×10^8 3.5×10^4
2727	1.8×10^4	2.9×10^6 8.8×10^5
3527	1.0×10^4	1.8×10^7 1.0×10^3

Five first day fifth instar larvae were injected with conidial suspensions of different concentrations from different isolates. The larvae were then incubated at 25°C ± 1°C and the mortality at 72 hours recorded. The work was repeated three times (n = 15). Isolates959,1558 and1629 caused insufficient mortality at 72 hours for probit calculation.

The method used to calculate LC_{50} and the 95% probability limits was that given by Finney (1964) but he warns against attaching too much importance to the probit itself for statistical analysis. Probit calculation converts the sigmoidal line of % kill into a straight line which can be used for extrapolation

Table 5 shows the LC_{50} for larvae 96 hours after injection with *B. bassiana* conidiospores.

Table 5 Concentration of *B. bassiana* conidia from different isolates which caused 50% mortality(LC_{50}) of *M. sexta* larvae 96 hours after injection.

Isolate	LC_{50}	95% Limits	Probit	χ^2	D.F.
304	2.6×10^4	3×10^5 2×10^3	0.85 ± 0.21	2.3 (a)	3
1007	1.2×10^5	1.7×10^5 8.7×10^4	3.3 ± 0.24	19.2 (c)	2
1122	5.4×10^5	7.4×10^5 3.9×10^5	3.8 ± 0.25	0.08 (a)	3
1315	1.0×10^4	7.2×10^6 1.5×10^1	0.43 ± 0.20	8.8 (b)	3
1484	1.3×10^5	2.2×10^5 7.8×10^4	1.85 ± 0.20	0.29 (a)	3
1886	6.8×10^5	9.9×10^5 4.6×10^5	2.82 ± 0.23	1.48 (a)	3
2417	1.6×10^6	1.7×10^8 1.4×10^2	0.20 ± 0.16	1.71 (a)	4
2727	4.6×10^5	6.8×10^5 3.2×10^5	2.6 ± 0.21	15.4 (c)	4
3527	4×10^2	2.8×10^8 0	0.59 ± 0.31	2.0 (a)	4

n = 45

Median lethal concentration of fungal conidia causing mortality at 96 hr with confidence limits and probability.The χ^2 test indicates the amount of agreement between the theoretical and observed results (goodness of fit).

(a) good fit (0.1>p)

(b) poor fit (0.5>p>0.1)

(c) very poor fit (p>0.5)

2.3.3 Change in larval weight gain after treatment with spore suspensions

a) Immersion in spore suspensions.

The weight gain of larvae which had been immersed in different spore suspensions of all twelve isolates showed remarkably similar trends. In every case except 1886, the control insects reached a greater weight gain at 96 hours after treatment than the experimental insects, even when spore concentrations were low.

In the case of 1886, a significant ($p<0.01$) difference in larval weight gain occurred with spore concentrations of 2.3×10^6 per ml, but not with lower doses.

For each isolate, four different spore concentrations had been used and in most cases weight gain decreased as spore concentration increased. In some cases, where the concentrations were close together, there was an interchange of slope between two adjacent concentrations, but the trend of weight gain overall was dictated by spore concentration.

Although the slope of weight gain against time is very similar for the different isolates in 304, 1007, 1484, 1866 and 2727, the slope for weight gain of the larvae treated with the highest spore concentrations flattens out at 72 h post treatment. Fungal development within the insect might be responsible for this. The curve in weight gain for the control insects rises most steeply at this point.

With these isolates at 96 hours, even the insects treated with the lowest spore concentrations have a lower weight gain than the controls.

Figure 1 of larval weight gain after immersion in spore suspensions of isolate 304 is typical of the results. Figures of the other isolates are to be found in the appendix. Figure 2 shows the relationship between spore concentration and weight gain at 96 hr after treatment of 5th instar *M. sexta* larvae immersed in a suspension of spores of *B. bassiana* Isolate 304.

Figure 1

Figure 1 shows the effect of immersion in increasing doses of spores of *Beauveria bassiana* isolate 304. Conidiospores were harvested from culture on SDAY, washed and suspended in sterile distilled water. Concentrations were counted with a Neubauer haemocytometer.

First day fifth instar *M. sexta* larvae were weighed and immersed for 10 seconds in conidial suspensions of isolate 304.

Five different concentrations of conidia in sterile distilled water were used with five larvae in each case. The larvae were incubated at 25°C ± 1°C and were weighed at 24 hour intervals.

Control insects were immersed in sterile distilled water. The work was repeated 3 times for each spore concentration giving n = 15.

Analysis of variance (ANOVA) showed that the weight gain of insects treated with doses of 9.3×10^5 and 2.6×10^6 spores per ml of isolate 304 differed significantly ($p < 0.05$) from the control from 24 hours onwards.

Treatment with lower concentrations of the same spores did not cause significant differences in insect weight gain.

FIGURE 1. Larvae immersed in Isolate 304

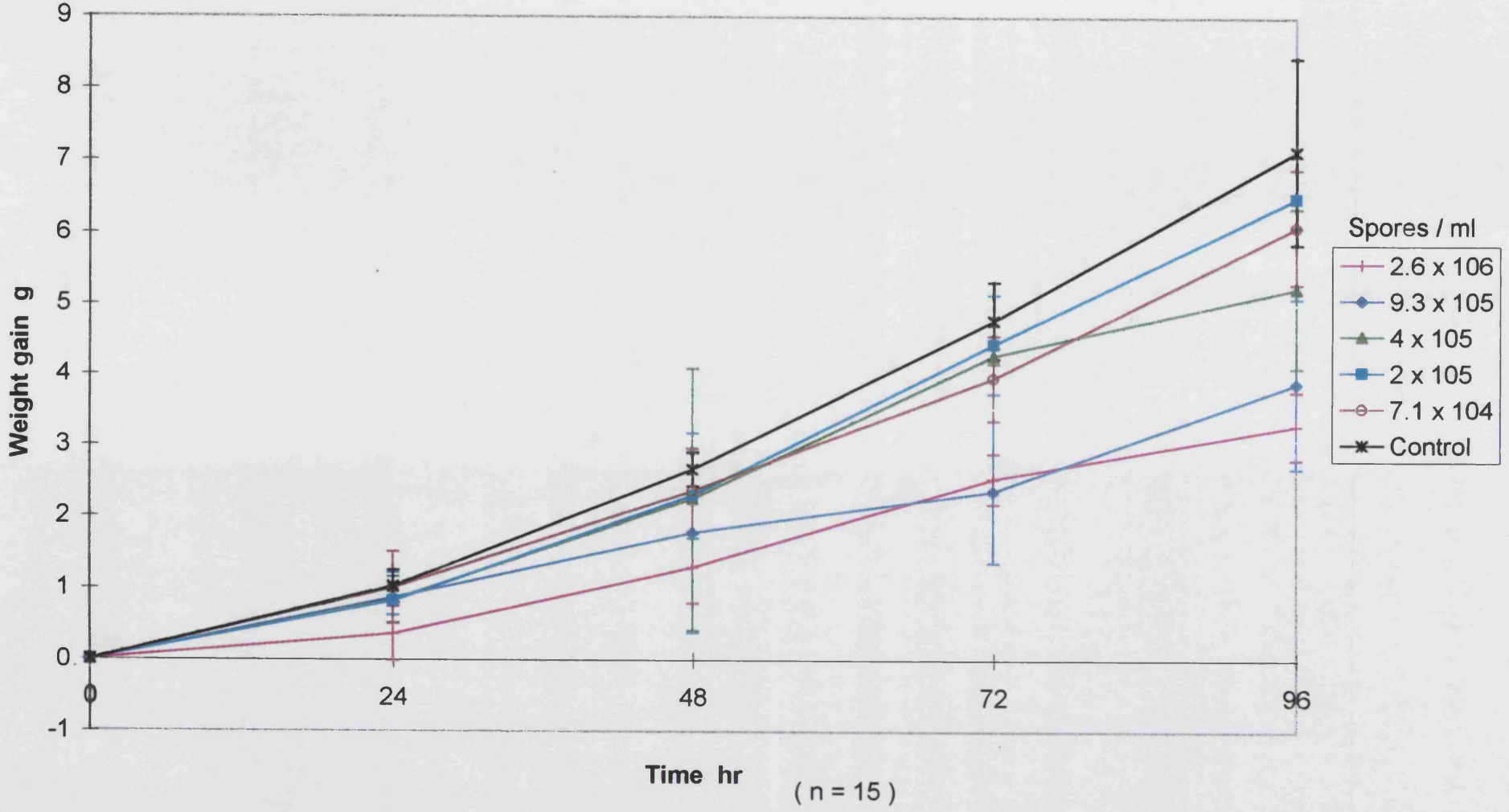
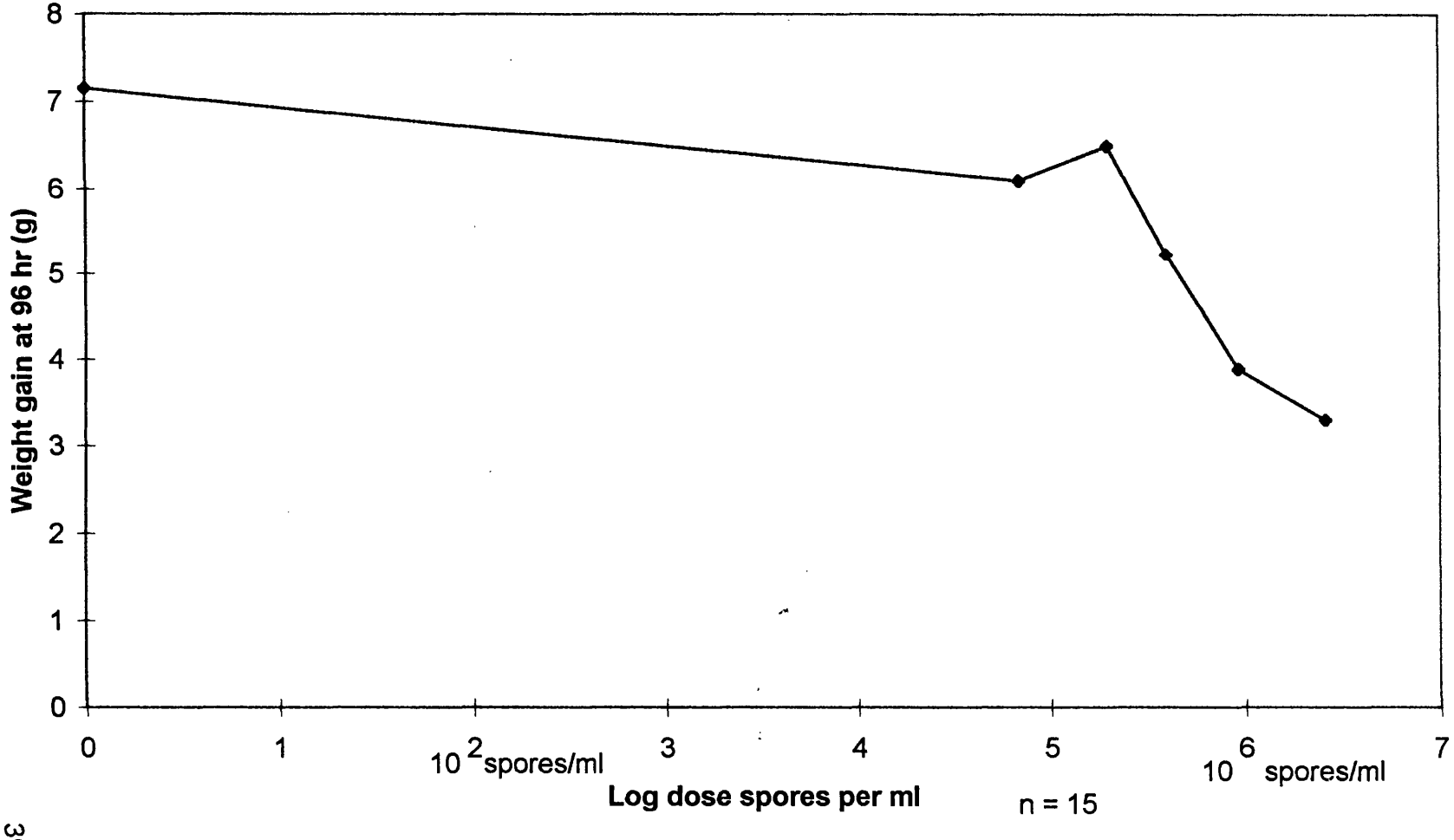


Figure 2 Effect on larval weight gain of varying doses of isolate 304 spore suspension

This figure shows the weight gain in grams of fifth instar *M. sexta* larvae 96 hours after 10 second immersion in different doses of *B. bassiana* isolate 304 conidiospore suspension.

Log of dose versus weight gain at 96 hours.

FIGURE 2 Effect of varying dose on weight gain, larvae immersed in Isolate 304



b) Injection of spore suspensions

The spores of isolates injected into the larvae caused effects which broadly fell into two groups. One group, which included isolates 959, 1558, 1886 and 2417, allowed the larvae to steadily gain weight, but at a slower rate than the control.

Figure 3 of larval weight gain after injection with isolate 1558, illustrates this group. The insects gained weight continuously and there were no abrupt changes in the rate of weight gain that might be associated with toxic metabolite production.

These isolates are among the least pathogenic of those tested. As had been found previously (Table 2) there was only limited mortality during the 96 hours of the experiment. Thus mortality at 96 hours was 66 % (Isolate 959); 33 % (Isolate 1558); and 87 % (Isolate 2417) in these experiments.

The second group of isolates (304, 1007, 1122, 1315, 1484, 1629, 2727, 3527) had much more serious effects on insect weight gain. In this group, the injected larvae gained weight for a limited time, after which weight gains levelled off. For isolates 1315 and 3527 the lack of weight gain occurred after 24 hours, with a very slight upward trend at 96 hr. For isolates 2727 and 304 the levelling off occurred at 72 hours and there was no compensating upward trend at 96 h. Figure 4 illustrates this for isolate 2727. Data for some of the other isolates of this group are in the Appendix V (pages 225-227).

The amount of weight gain and the rate at which it was gained were strongly influenced by the injected spore concentration, the greater the concentration the less the larval weight gain.

Figures 3, 4 and 6

Five first day fifth instar larvae were weighed and then injected with 10µl of spore suspensions of isolate 1558 (Fig.3), isolate 2727 (Fig.4) or isolate 1484 (Fig.6).

Four different conidial concentrations in distilled water were used for each isolate and five larvae injected in each case. The larvae were incubated at 25°C ± 1°C and weighed at 24 hour intervals.

Control insects were injected with ten microlitres of sterile distilled water.

The work was repeated three times with five larvae for each conidial concentration of each isolate giving n = 15.

Figure 5

Figure 5 shows the variation in weight gain of larvae against the log of the dose of injected conidiospores of isolate 2727. The conditions are the same as Figure 4.

The R value of -0.78 suggests that lack of larval weight gain is highly dependent on the concentration of conidia injected.

FIGURE 3. Larvae injected with 10 µl of Isolate 1558

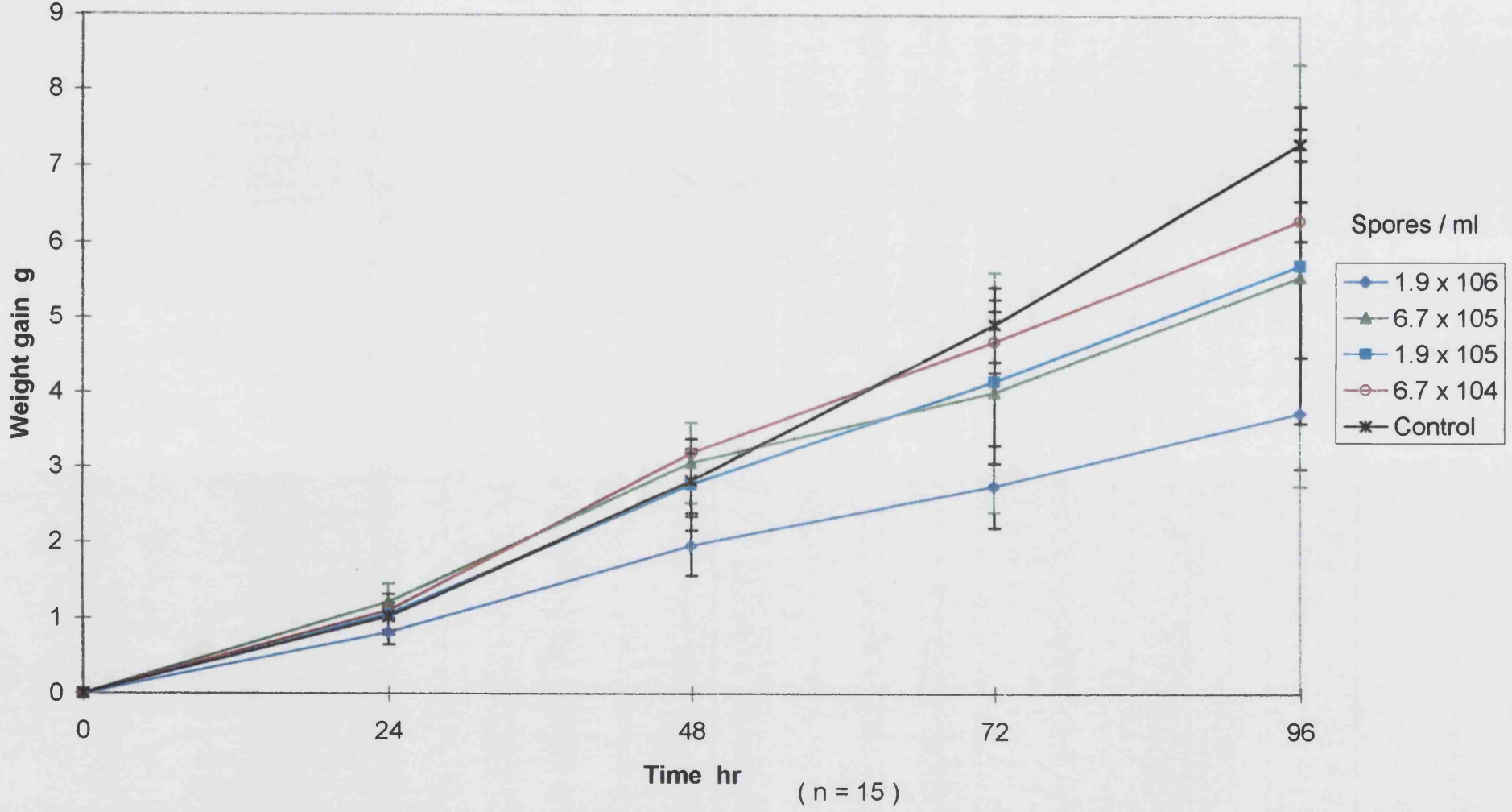


FIGURE 4. Larvae injected with 10µl of Isolate 2727

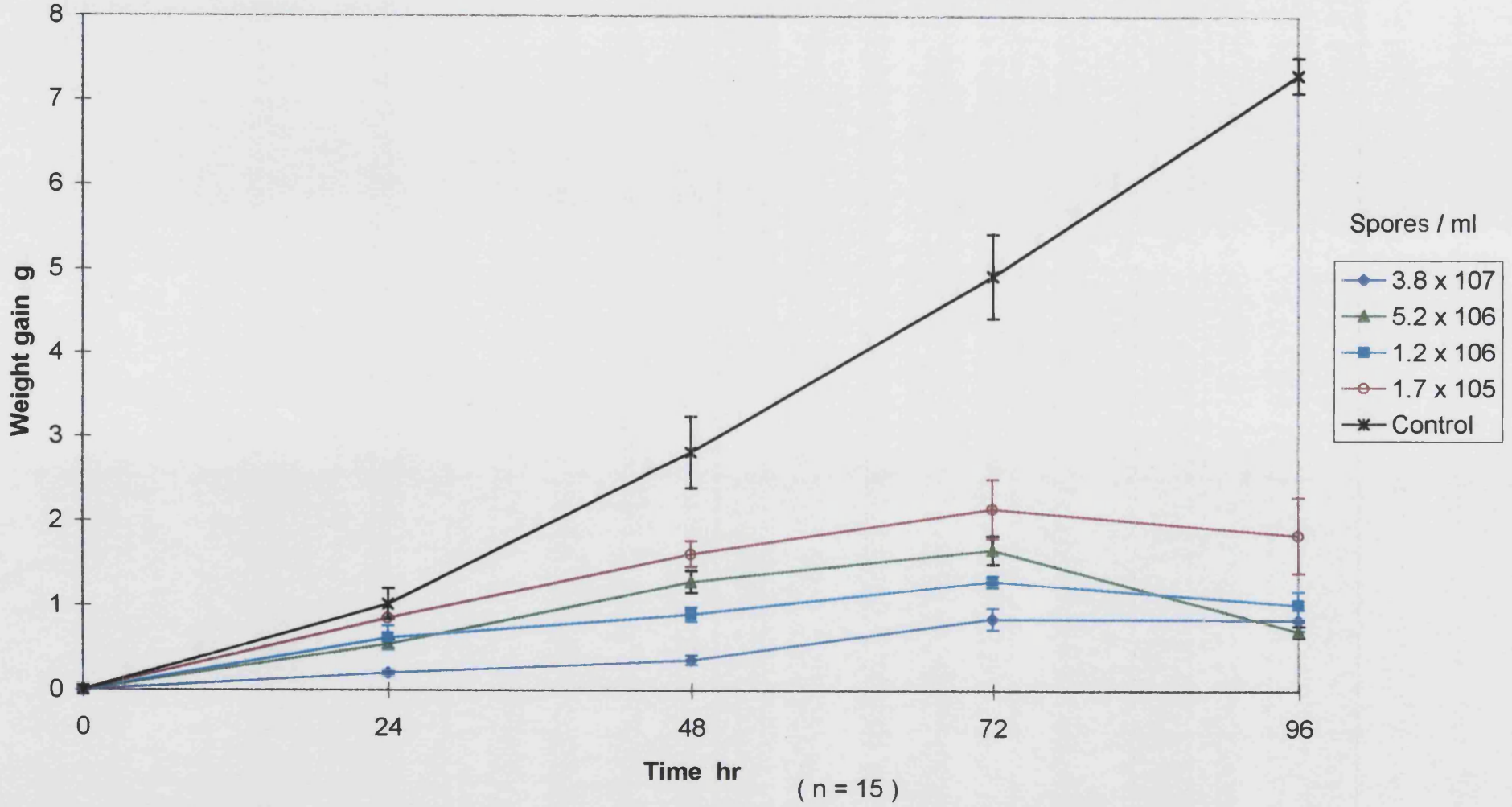
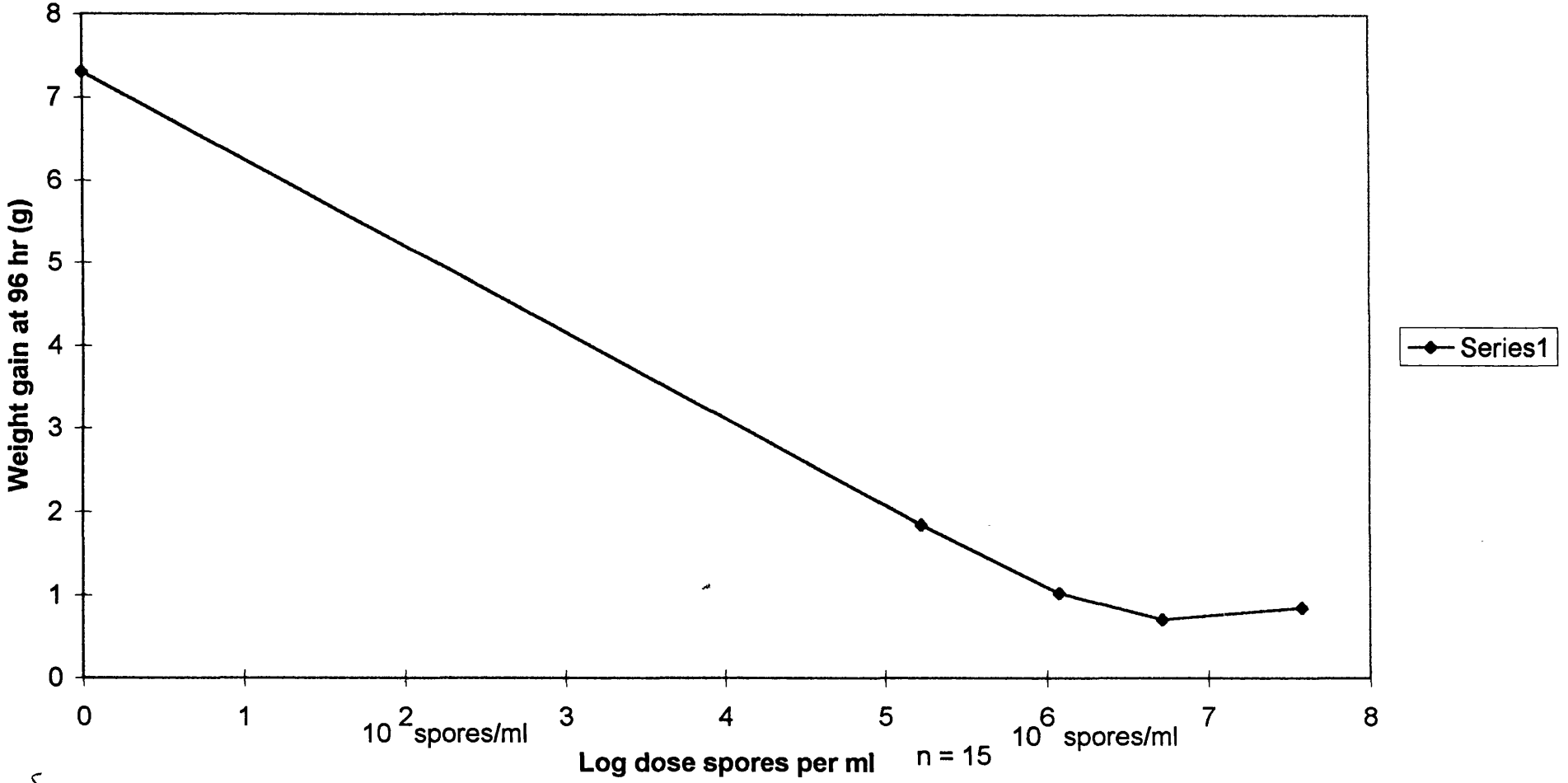


FIGURE 5. Effect of varying dose on weight gain, larvae injected with 10µl of Isolate 2727



c) Effect of spore concentration on larval weight gain

The adverse effect of spore injection on larval weight gain was dose-dependent. The relationship between 96 hr weight gain and the concentration of injected spores for Isolate 2727 is shown in Figure 5.

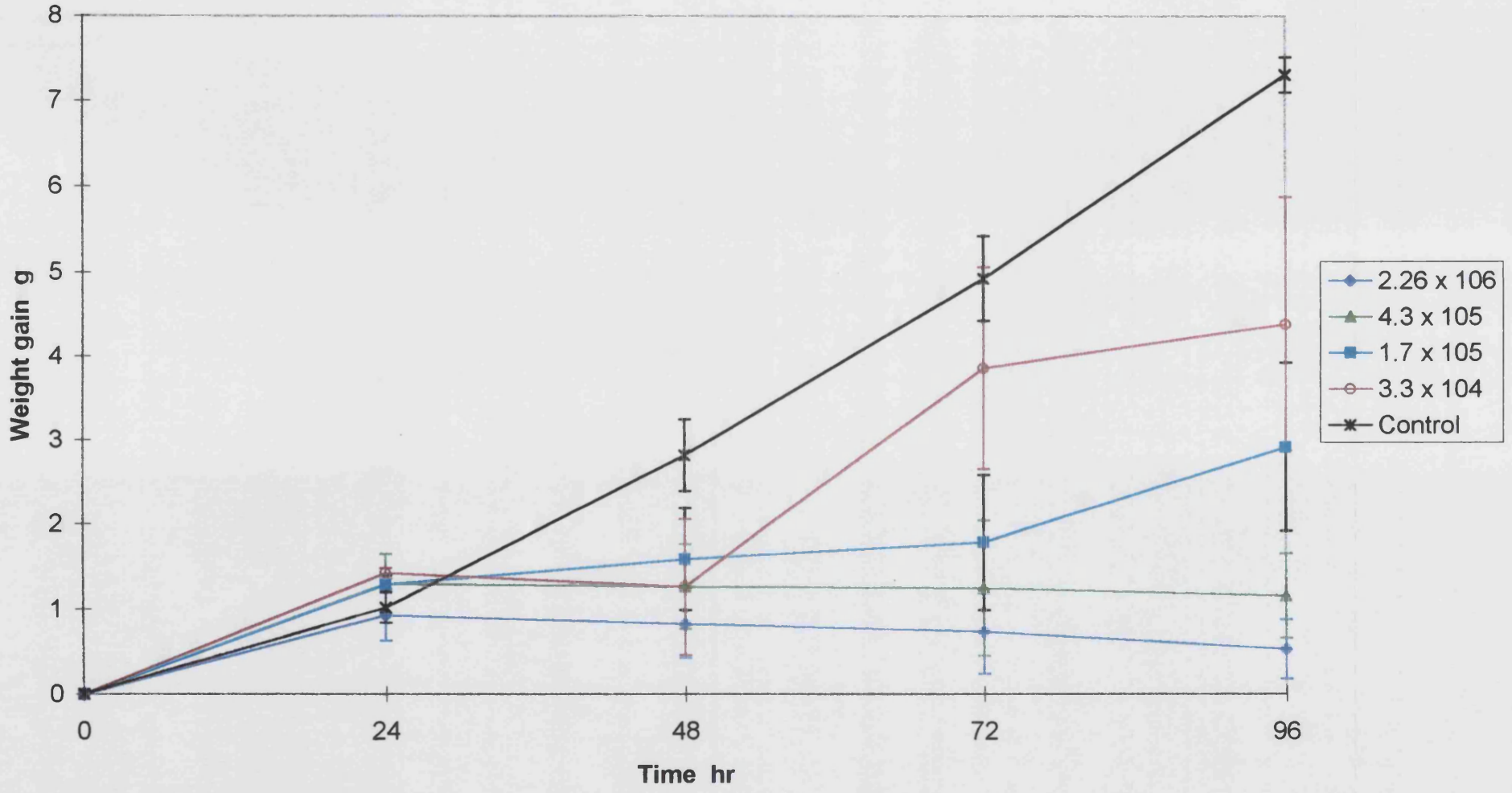
With all the isolates the effect of spore concentration was evident. Even where weight gain was continuous for all the larvae, as in 1558, the higher the inoculation rate, the less the growth. In the use of isolates 1007 and 1629 the larvae injected with the lowest spore concentration (5×10^4 spores per ml) continued to gain weight whilst higher concentrations caused levelling off and at 24 hours insects injected with 5.5×10^6 spores per ml started to lose weight. This may reflect the fact that these isolates cause high mortality at 96 hours.

Isolates 1484, 1886 and 1122 all illustrate the influence of spore concentration. Larvae injected with doses above 4×10^5 spores per ml showed little growth 24 hours after injection. Those injected with less than 6×10^5 spores per ml were able to gain weight, though in the case of isolate 1886 larvae started to lose weight at 72 hours.

Figure 6 of larval weight gain after injection with spores of 1484 illustrates the effect of different concentrations of inoculum of this group of isolates.

A comparison of larval weight gain and LC_{50} (2.3.2) using spores of different isolates shows that pathogenicity and lack of weight gain are not always linked.

FIGURE 6. Larvae injected with 10µl of Isolate 1484



Briefly, group (i) isolates (959,1558,1886 and 2417) allowed larvae to gain weight continuously, but slowly, over 96 hr at all doses;

group (ii) isolates (304,1007, 1122, 1315, 1484, 1629, 2727 and 3527) allowed larvae to gain weight for only a short time after which they remained the same, or lost weight, at all doses.

The main single factor to emerge from the larval injection was that all the isolates caused a reduced rate of weight gain. Weight gain was closely linked to spore concentration and in some cases a change in slope suggested that metabolic products from the fungi could be detrimental to larval weight gain.

2 .3 .4 Faecal weight from larvae immersed in spore suspensions of different isolates

All the *B. bassiana* isolates caused reduced production of faeces by the treated insects (Table 6), presumably reflecting a decreased intake of food, or the ability ro utilise it. When the results were ranked isolates 304, 1484, 1122 and 2727 had the largest effect on faeces production at 96 hours.

Immersion in spore suspensions of Isolates 304, 1122 and 2727 had also caused the largest reduction in larval weight gain. Immersion in spore suspensions of 1315 and 1629 had the least effect on faeces production.

Table 6 Total dry weight of faeces produced by larvae from 0 to 96 hours after immersion in spore suspensions of different isolates

Isolate No.	Concentration spores per ml	Weight of faeces (g)	Standard error of mean
304	4×10^6	3.69	0.25
959	4×10^6	4.48	0.45
1007	4×10^6	4.39	0.08
1122	1×10^6	3.94	0.47
1315	2×10^6	5.18	0.41
1484	2×10^6	3.88	0.49
1558	Data	not	available
1629	5×10^6	5.14	0.04
1886	4.5×10^6	4.04	0.51
2417	9×10^6	4.31	0.37
2727	9.5×10^6	3.94	0.67
3527	1×10^6	5.04	0.48
Control	Mean of 4	5.31	0.91

(faeces from 3 × 5 larvae)

Five first day fifth instar larvae were immersed for 10 seconds in spore suspensions of the different isolates. The larval faeces of each group were collected daily and after 96 hours of incubation were combined and dried to a constant weight.

All the treated larvae produced less faeces than the control, with isolates 304, 1484, 1122 and 2727 having the most effect. Isolates 1315, 1629 and 3527 had the least effect.

There is evidence to suggest that whilst the effect varies from isolate to isolate an increasing spore concentration reduces faeces production. In both the most effective (304) and least effective (1315) isolates the amount of faeces produced by the five test larvae increased with immersion in a reduced spore concentration. As with larval weight gain, correlation between the concentration of spores used and faeces producution was negatively correlated. The higher the rate of inoculum the smaller the amount of faeces produced, with spores of isolate 304 the R value was -0.524 and with isolate 1315 R= -0.357.Even if the spores of these isolates were not pathogenic at low concentrations they were still able to have some effect on the insect's feeding or digestive processes. The reduced faeces production may have been due to a reduction in larval feeding, malaise or reduced digestion. Alternatively it may have been due to the fungal capture of nutrients from the insect haemolymph.

Table 7 Effect of immersion in different spore concentration on larval faeces production

Isolate	Concentration per ml	Faeces dry weight in g
304	4×10^6	3.69 ± 0.25 (SEM)
304	8×10^5	3.47 ± 0.31
304	4×10^5	4.31 ± 0.08
304	8×10^4	4.50 ± 0.37
1315	2×10^6	5.18 ± 0.41
1315	2.6×10^5	5.43 ± 0.51
1315	1.3×10^5	4.73 ± 0.48
1315	2.6×10^4	6.14 ± 0.08
Control		6.17 ± 0.91

(faeces from 3 × 5 larvae)

Five first day fifth instar larvae were immersed in the different concentrations of spore suspensions of isolates 304 and 1315. The larvae were incubated for 96 hours and the faeces of each group collected daily, combined and dried to a constant weight.

2.3.5 Ability of conidiospores of different isolates to germinate on and penetrate the larval cuticle

a) Germination on cuticle

Three replicate tests of germination of spores (after 24 hours) on the outside of cuticle were carried out. Over 200 spores were counted in each case but the speed of germination and pathogenicity of isolates (in terms of LT^{50}) towards *M. sexta* larvae after immersion did not appear to be linked.

Table 8 shows the ratio of germinated to non-germinated spores 24 hours after inoculation onto the cuticle surface.

Table 8

Germination of spores after 24 h. on cuticle surface

Isolate Number	Non-germinated	Germinated	Ratio	LT50
304	387	288	1.3:1	58h
959	527	313	1.7:1	87h
1122	1256	54	23:1	77h
1629	1572	52	30:1	81h
2727	1929	106	18:1	67h

(actual numbers of spores)

50µl of spore suspension of 5×10^6 per ml of each isolate was applied to five first day fifth instar larval cuticles. These were incubated for 24 hours at 25°C after which the germinated and non-germinated spores were counted using phase contrast. The work was repeated 3 times.

b) Penetration of cuticle

Inoculated cuticle was removed from the culture media (SDAY) after different periods of time (24h, 48h, 72h, 96h). It became apparent from the print of the cuticle on the media that spores of all the isolates tested were able to penetrate the surface in less than 24 hours.

Isolate 1629, after penetrating the cuticle, characteristically produced pink pigmentation (oosporein), which diffused into the culture medium.

2.3.6 Examination of the haemolymph of larvae immersed or injected with spore suspensions

The haemolymph of treated insects was examined in order to assess the development of the fungus within the insect and the reaction of the larval haemocytes to fungal challenge. Treated insects were discarded after the haemolymph had been withdrawn. To withdraw haemolymph from the same insect at successive stages would have given a profile of infection, but after the first withdrawal, side-effects from trauma would alter results.

Aliquots of haemolymph were treated with F.I.T.C. wheat germ agglutinin, or Calcofluor and examined under a microscope with U.V. light. Although lectin-labelled fungal fragments, blastospores and hyphal bodies fluoresced under U.V. light, the inability to see haemocytes, or to use a haemocytometer to quantify results meant that this was a limited method and a light microscope with phase contrast gave more useful results.

Haemocytes, blastospores and hyphal bodies were counted using a haemocytometer and a light microscope with phase contrast (see Table 9). At 24 hours after treatment, the number of haemocytes in the haemolymph from both immersed and injected insects varied between 3×10^4 per ml and 3.9×10^5 per ml. The control value was 1.5×10^5 per ml. Three isolates (959, 1007 and 1122) had counts less than half of the control. This was true for both injection and immersion treatments. Haemocytes from insects injected with isolate 1315 showed some rounding up and loss of filopodia. At 24 hours there was no difference in the number of haemocytes in samples from injected or immersed larvae, but at 72 hours there was a ten times difference in the number of haemocytes from larvae treated with spores of isolates1484,1886 and 3527.

At 24 hours there was no evidence of the conidiospores of any of the isolates which had been injected. However, haemolymph from insects injected with two of the isolates, 1484 and 1315, both contained blastospores at 3.3×10^4 per ml and 1×10^4 per ml respectively. These two isolates may be able to develop more rapidly than the others tested.

At 48 hours, total haemocyte counts from infected insects were not significantly reduced ($p>0.05$). The number of haemocytes from the haemolymph of larvae injected with 6 of the isolates (1484, 1558, 1886, 2417, 2727 and 3527) was less than the control value, but the difference was not significant, whilst the number of haemocytes in the control insects remained constant. At this time,

although some of the haemocytes in the treated insects showed rounding up, it was not a consistent feature.

At 48 hours, haemolymph from insects injected with isolates 304, 1315 and 1484 contained hyphal bodies at 1.7×10^5 per ml, 4.6×10^5 per ml and 6×10^4 per ml respectively, whilst haemolymph from 1558 and 1629 injections had 1×10^4 hyphal bodies.

Plate 1 (after Table 9) shows haemolymph from first day fifth instar *M. sexta* larvae 72 hours after injection with spores of *B. bassiana* isolate 2727.

Table 9

Table 9 shows the effect of injecting (10µl of 1 × 10^6 spores per ml), or immersing (10 secs in 1 × 10^6 spores per ml suspension) first day fifth instar larvae in conidial suspensions. The effects of the twelve different isolates on the larval haemolymph were compared.

After treatment the larvae were incubated at 25° C ± 1° C with a 17/7 hour light/dark regime. At 24 hour intervals insects were sacrificed and the haemolymph allowed to drip into 1.5 microcentrifuge tubes containing several crystals of phenylthiourea. Haemolymph from the tubes was gently mixed and then placed on a Neubauer haemocytometer. Blastospores, hyphal bodies and haemocytes were scored using an Olympus microscope at × 400 magnification with phase contrast.

Larval treatments (immersion and injection) were repeated 3 times with each isolate (n = 3).

Table 9. Haemolymph of *M. sexta* larvae treated with 10^6 *B. b.* spores per ml by injection (10 µl) or immersion

Isolate	Treatment	24 hr			48 hr			72 hr			96 hr		
		Blasto-spore	Hyphal body	Haemo-cyte	Blasto-spore	Hyphal body	Haemo-cyte	Blasto-spore	Hyphal body	Haemo-cyte	Blasto-spore	Hyphal body	Haemo-cyte
304	Inject	0	0	1.6×10^5	1×10^4	1.7×10^5	1.5×10^5	1×10^4	0	6×10^4	—	Dead	—
304	Immerse	0	0	9.3×10^4	0	0	1.7×10^5	0	0	1.5×10^5	0	0	6×10^4
959	Inject	0	0	5×10^4	0	0	5.1×10^4	0	1.2×10^5	2×10^4	—	Dead	—
959	Immerse	0	0	3×10^4	0	0	1.5×10^5	0	0	6×10^4	0	0	7×10^4
1007	Inject	1×10^4	0	5×10^4	0	0	1×10^5	0	0	1×10^4	—	Dead	—
1007	Immerse	5×10^3	0	6.6×10^4	0	0	1×10^5	0	0	2×10^4	0	0	9×10^4
1122	Inject	0	0	5×10^4	0	0	9.1×10^4	0	1×10^4	1×10^4	0	1×10^4	0
1122	Immerse	0	0	6.6×10^4	0	0	1.3×10^5	0	0	2×10^4	0	0	5×10^4
1315	Inject	0	0	2.6×10^5	0	4.6×10^5	1.2×10^5	0	1×10^4	0	1×10^4	1×10^4	0
1315	Immerse	0	0	1.4×10^5	0	0	1.1×10^5	0	0	1.3×10^5	0	0	2.3×10^5
1484	Inject	3.3×10^4	0	9.6×10^4	0	6×10^4	3×10^4	0	1×10^4	1×10^4	—	Dead	—
1484	Immerse	0	0	1.1×10^5	0	0	1.3×10^5	0	0	1.8×10^5	0	0	1.1×10^5

n = 3

Table 9 (cont.) Haemolymph of *M. sexta* larvae treated with 10^6 *B. b.* spores per ml by injection (10 µl) or immersion

Isolate	Treatment	24 hr			48 hr			72 hr			96 hr		
		Blasto-spore	Hyphal body	Haemo-cyte	Blasto-spore	Hyphal body	Haemo-cyte	Blasto-spore	Hyphal body	Haemo-cyte	Blasto-spore	Hyphal body	Haemo-cyte
1558	Inject	0	0	2.3×10^5	0	1×10^4	4×10^4	0	0	6.6×10^4	0	0	1×10^5
1558	Immerse	0	0	1.3×10^5	0	0	8×10^4	0	0	7.6×10^4	0	0	1.9×10^5
1629	Inject	0	0	8×10^4	4.2×10^5	1×10^4	1×10^5	0	6.3×10^4	1×10^4	—	Dead	—
1629	Immerse	0	0	1.8×10^5	0	0	7×10^4	0	0	9×10^4	0	0	5.7×10^5
1886	Inject	0	0	3.9×10^5	0	0	4×10^4	0	2×10^4	5.1×10^4	—	Dead	—
1886	Immerse	0	0	2.3×10^5	0	0	1.1×10^5	0	0	4.1×10^5	0	0	1.3×10^5
2417	Inject	0	0	1.3×10^5	0	0	5.3×10^4	0	1×10^4	0	—	Dead	—
2417	Immerse	0	0	1.4×10^5	0	0	9×10^4	0	0	1.6×10^5	0	0	1.3×10^5
2727	Inject	0	0	9.6×10^4	0	0	3×10^4	1×10^4	2.1×10^5	3×10^4	—	Dead	—
2727	Immerse	0	0	1.4×10^5	0	0	7×10^4	0	0	9×10^4	0	0	7.3×10^5
3527	Inject	0	0	1.1×10^5	0	0	4×10^4	0	3×10^4	1×10^4	—	Dead	—
3527	Immerse	0	0	1.3×10^5	0	0	1.4×10^4	0	0	1.2×10^5	0	0	7×10^4
Control	Inj/Imm	0	0	1.5×10^5	0	0	1.6×10^5	0	0	1.1×10^5	0	0	1.6×10^5

n = 3

PLATE 1

Hyphal bodies ↑ ↑ Rounded haemocytes ↑↑

Haemolymph of *M. sexta* larvae 72 hours after injection with spores of *B. bassiana* isolate 2727.

Rounded up haemocytes, lacking filopodia, are evident (1.9×10^5 per ml).

Hyphal bodies (short fragments of hyphae), with developing septa, are numerous (7.6×10^5 per ml).

There are no conidiospores.

At 72 hours insects injected with conidiospores of isolates 1122, 1558, 1629 959 and 1886 gave haemolymph which was brown-green instead of the normal blue-green colour, and which was less viscous than the control. The haemocyte counts from injected larvae had dropped further still and counts from larvae injected with conidiospores of the eight isolates 959, 1007, 1122, 1484, 1629, 1886, 2727 and 3527 were all less than half of the control value. Of the immersion treatments, spores of isolates 1007 and 1122 both produced very low values of haemocyte counts. Control values were still similar to those seen earlier. Larvae treated by injection with spores of the isolates 304, 2727 and 3527 still had blastospores in the haemolymph, whilst all except 304, 1007 and 1558 and the control contained between 1×10^4 and 1×10^5 hyphal bodies per ml.

At 96 hours all of the injected larvae were either moribund, or had died. Where haemolymph was obtainable from live insects very few blastospores or hyphal bodies remained. The cadavers had become mycosed and mycelium had started to grow out of them

In the immersed insects only haemolymph from larvae dipped in spore suspensions of isolate 2727 had an appreciable number of blastospores at 96 hours, and this only in one of the three replicate trials. This suggests that this isolate may be able to breach the insect cuticle more rapidly and effectively after immersion than the other isolates.

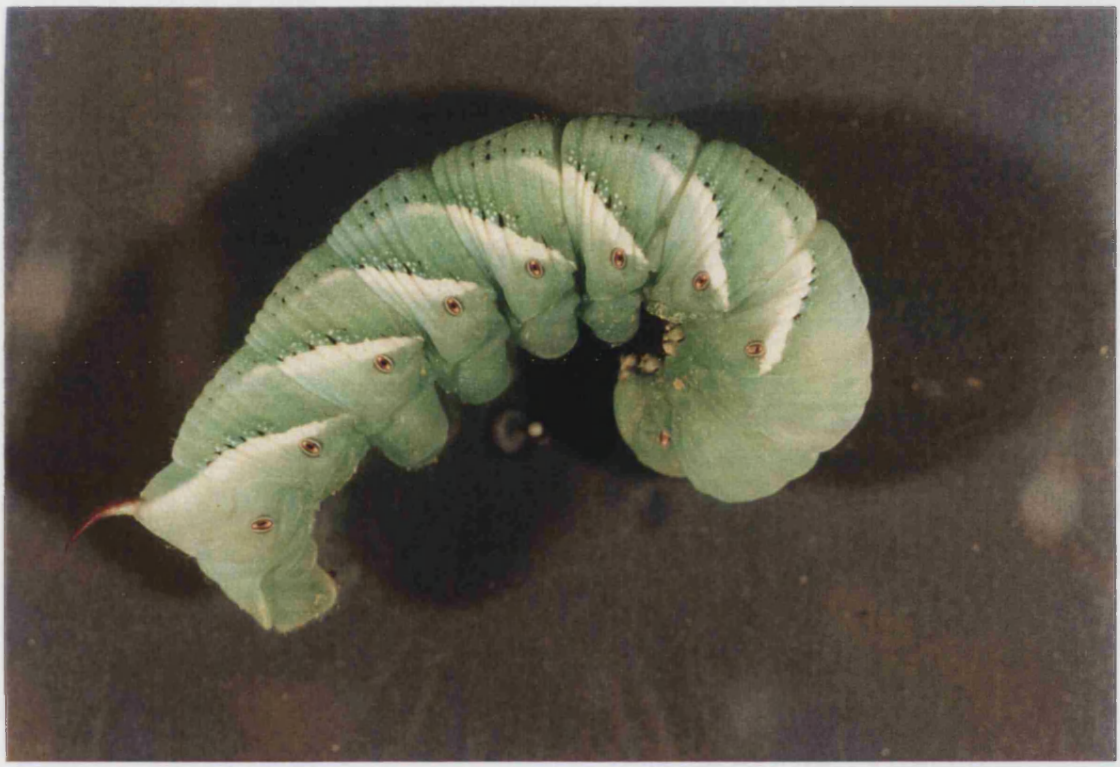
Numbers of haemocytes from insects immersed in spore suspensions were comparable with the control, but in three cases, isolates 1315, 1886 and 2417, the haemocytes were rounded up, and in some cases appeared to have aggregated.

From an examination of the haemolymph it appears that all the isolates investigated affect the larvae within 96 hours of injection. Since the death of larvae immersed in spore suspensions of these isolates takes seven days or more, one would expect fungal development within the haemolymph to be slower after immersion than after injection.

2.3.7 Changes in appearance of *M. sexta* larvae.

Normal *M. sexta* larvae are blue-green in colour. Their body colour may become an intense green if they are fed on tomato leaves, but when fed on artificial diet (as was the case in these experiments) they remain blue-green during the feeding period of the fifth instar becoming paler after wandering before they pupate. The white dorsal markings and bright yellow spiracles with dark centres remain throughout the duration of the fifth instar.

PLATE 2



Untreated *M. sexta* fifth instar larva × 2

PLATE 3

***M. sexta* fifth instar larva (× 2) 72 hours post injection with spores of Isolate 2417 (10 µl of 1 × 10^6 spores per ml)**

Larvae which had been treated with some *B. bassiana* isolates by injection developed a pale pink colour at 72 hours post injection which deepened until the insect died. These isolates also produce a pink colour when grown on SDAY or in Czapek-Dox liquid media. The pigment is probably oosporein which is produced by some isolates of *Beauveria bassiana* and also *Verticillium lecanii.*(El Basyouni, Brewer and Vining, 1968; Eyal, Mabud, Fischbein, Walter, Osborne and Landa, 1994).

Isolates 1315, 1484, 1629, 2727, 2417 and 1122 all caused pink/red coloration in larvae by 72 hours after spore injection, whilst Isolates 1484 and 2727 also caused the coloration after larvae were immersed at doses of more than 1 × 10^6 spores per ml at 7 days. Since this fungal pigmentation occurs when colonies are actively growing on SDAY it suggests that the isolates which are able to produce it in the larvae must also be actively growing.

After death the white fungal mycelia outgrow and cover the pink pigmented larvae.

In all the infected larvae, a sequence of change in coloration of the cuticle occurs irrespective of the effect of fungal pigment production.

At the earliest point, usually about 48 h before death, the larvae show small brown patches mainly on the dorsal surfaces of the rear segments. There is no particular pattern to this melanisation, it does not for example occur especially around the spiracles or along the inter-segmental membranes.

PLATE 4

***M. sexta* larva (× 1.5) showing melanisation 48 hr prior to death, after injection with 10μl of 1 × 10^6 spores per ml. of isolate 304.**

melanised patches

At 24 h prior to death, when the larva may have become sluggish, the darkened patches on the cuticle become larger and more numerous and include the whole body and head, but the white dorsal markings and the spiracles are still clearly visible.

After death a fine white mycelial growth appears firstly over the dorsal surface of the larvae and most notably along the white stripes. The head, the last abdominal segment, the under side of the insect and the legs are not immediately affected. Finally, usually at least 72 h after death, but depending on the humidity, the whole insect, apart from the head, is covered with white mycelial growth, which later produces conidia, thus enabling the fungus to continue the infection process.

PLATE 5

***M. sexta* larva (× 2) with outgrowth of *Beauveria bassiana* mycelia 72 hr after death**

2.4 Discussion

The effects of a pathogen on its host are not always the most obvious. This study sets out to compare the effects of different *Beauveria bassiana* isolates on the host insect *Manduca sexta.*

Bidochka and Kachatourians (1990) define fungal pathogenesis as the biochemical, physiological and genetic processes during insect infection and disease formation, whilst virulence is the ability of an entomopathogenic fungus to cause death.

2.4.1 Immersion in spore suspensions

B. bassiana is such a well-known and widespread insect pathogen that it is surprising to find that there are relatively few published records of the effects it has on its host. Gupta *et al* (1994) looked at the mortality of *G. mellonella* after immersion in *B. bassiana* spore suspensions and liquid culture supernatants. Although these methods used five different isolates they did not compare the effects of administering spores by immersion with injection. Gupta *et al.* (1994) considered LT_{50} as a means of describing strain virulence and suggested that onset of mortality and rate of mortality might give more useful comparisons between isolates than LT_{50}.

In the present study of *B. bassiana* against *M. sexta,* the assessment of the percentage mortality against time indicated three highly pathogenic isolates (304, 2727 and 3527) which were effective 48 hours post injection. It also highlighted an isolate (1558) with poor virulence even at six days. The effect of immersion of larvae in spore suspensions showed considerable differences between isolates with four having no lethal effect at all after 7 days and larvae pupating normally.

Gupta used some isolates of *B. bassiana* which had an LT_{50} of more than 7 days. When 5th instar *M. sexta* larvae are used, an LT_{50} of seven days at the most can be indicated since the larvae would normally have reached pupation by then.

From the isolates tested it appeared that spore concentration played a major part in the effect of *Beauveria* on the insect host. Jones, Grace and Tamashiro, (1996), working on the pathogenicity of *B. bassiana* to the termite *Coptotermes formosanus,* calculated the median lethal concentration of spores to achieve 50% mortality (LC_{50}). They showed that the LC_{50} varied between isolates and suggested that the strain with the lowest LC_{50} was the most virulent.

In the study of *M. sexta* larvae mortality was so low after immersion in *B. bassiana* spore suspensions that it was impracticable to calculate LC_{50} at 72 or 96 hours. However on injection the effects of concentration were evident and LC_{50} was calculated.

There was a considerable difference in the LC_{50} between isolates (see Table 5), with 3527 (4×10^2) the lowest at both 72 and 96 hours, whilst 2417 (1.6×10^8) was the highest, There does not appear to be any direct correlation between LT_{50} and LC_{50}. When ranked the concentration of spores did not appear to be linked with speed of kill, which suggests that isolates with a small LT_{50} might produce early toxic metabolites rather than killing the insects by mycosis. However 3527, with the lowest LC_{50} would, according to Jones theory, be the most virulent.

Few workers appear to have considered weight gain after treatment with pathogenic fungi in any detail. Samuels, Charnley and Reynolds (1988a) suggested that reduced weight gain was the first quantifiable effect of mycosis and appeared to be negatively correlated with the concentration of the inoculum, but there are no published papers which include work on daily weight gain of *M. sexta* larvae after inoculation with *B. bassiana* spores either by immersion or injection. A considerable amount of work has been carried out on cuticle-degrading enzymes (Gupta *et al.*, 1992; St.Leger, Joshi, Bidochka, Rizzo and Roberts, 1996a) and on inoculated insect haemolymph (Pendland *et al.*, 1993; Mazet, Hung and Boucias, 1994) but the effect of infection on daily weight gains appears to have been overlooked.

Weight gains after different types of treatment and at different spore concentrations can give a picture of the overall effect of the fungus on the insect and may indicate a reduced appetite, malaise, or an inability to digest food and mature. Gupta *et al.* (1992) found in *B. bassiana* a considerable fungal strain variability in cuticle-degrading enzyme production. This finding is echoed by the variation in the amount of weight gain of the insects immersion-treated with different isolates. The pattern of weight gain is the same, but the amount varies from one isolate to another.

In the twelve isolates tested insect growth rate was reduced in proportion to the concentration of the spores. This suggested that although growth continued in the treated insects it was subjected to a consistent limiting factor.

In isolates causing the highest mortality seven days after immersion (304, 2727 and 3527) there was a trend for the curve to level off at the highest concentrations at 72

hours. It is possible that the larvae were already beginning to be affected by mycosis at this time and this was reflected by lack of weight gain. By contrast, insects treated with isolates causing little mortality continued on the same weight curve until they reached the "wandering" stage.

The similarity of the growth curves of immersion-treated insects is striking. Whether the isolate is highly pathogenic or not it exerts an influence on larval weight gain which translates as a longer period before pupation and possibly a lower pupal weight.

Treated larvae failed to produce faeces at the same rate as the controls. As with weight gain, there was a considerable difference between isolates, but the insects treated with isolates which reduced weight gain most, also caused the greatest decrease in faecal production (304, 1484, 1122 and 2727). Kershaw (1993) suggested that reduced weight gain might be due to the invasive effects of fungus growth on the host physiology, as well as a reduction in food intake. This would provide an explanation for the effect of greater spore concentrations on larval growth.

The work carried out on the excised larval cuticle demonstrated that all the *B. bassiana* isolates tested were able to penetrate the cuticle. It seems probable that the difference between the isolates in their pathogenic success was due to other factors than their ability to penetrate the insect cuticle.

Some isolates might be more successful than others simply because they could grow more quickly within the insect. It is also possible that some isolates might be able to combat the insect's immune defences more rapidly, or more completely.

2.4.2 Injection of spores

The injection of a known concentration of spores may give a more precise measurement of their effect than immersion. Mollier, Lagnel, Fournet, Aloun and Riba (1994) injected *Galleria* larvae to determine the toxic activity of filtrates and Pendland *et al.* (1993) used injection in the study of evasion of the host defence by *B. bassiana.* Although many workers have treated insects by immersion the injection of spores into the larvae allows a known quantity of inoculum to be placed within the cuticle. It also circumvents the problem of fungal isolates unable to produce appressoria or isolates which do not produce cuticle-degrading enzymes. It may provide a means to distinguish between the enzyme activity of different isolates and it may overcome the cuticular barriers which some species of insects have against pathogenic fungi.

Larvae injected with different isolates and concentrations displayed different patterns of weight gain. *B. bassiana* produces a considerable range of metabolites and this may be reflected in the weight gain response. The picture is further complicated by the insect's cellular response to the fungal spores.

High doses of spores of isolates 304, 1484, 1629 produced weight loss; medium doses produced a small weight gain, but low doses produced a small weight gain until 72 hours, when the curve rose steeply and the insect appeared to have overcome the fungal effects. It may be that at this point with low doses the insect haemocytes are able to combat fungal development, or that the insect has been able to produce anti-fungal metabolites .

Isolates 1558 and 2417 appeared to cause dose-related weight gain, but always less than that of the control, indicating a growth-limiting effect which existed at low doses and continued until 96 hours post-injection.

Larvae treated with isolate 1886 continued to grow normally, albeit at a lower rate than the control, then, at 72 hours began to decline rapidly. Here, one might suggest that either the fungus has overcome the insect's defences, or it is able to produce new and more toxic metabolites, or even that the rate of development within the insect has increased and the fungus is able to produce larger amounts of the toxic metabolites than at first. Alternatively the insect's response to the fungal challenge could have been overwhelmed at this point.

One way to resolve some of the questions that these changes in weight gain pose is to look at changes at twenty-four hour intervals within the insect haemolymph. It may be that with low doses of spores the insect haemocytes are able to combat fungal development, but at high spore doses the haemocytes are overwhelmed.

Hung, Boucias and Vey (1994) showed that *B. bassiana* spores inhibited the spreading of haemocytes of *Spodoptera exigua* 24 hours after injection. Boucias *et al.* (1994) suggested that mycopathogens reduce the number of spreading haemocytes and inhibit the host's response to infection. He noted detectable alterations in haemocyte behaviour at 24 hours and a reduction in numbers at 48 hours, and suggested that some mycopathogens are able to evade and survive the host immune system.

In the present work, when the haemolymph of *M. sexta* larvae injected with different isolates of *B. bassiana* was examined 24 hours post-treatment, there was little evidence of a change in the shape of the haemocytes, only spores of isolate 1315 had

caused some rounding-up. However between injection and 72 hours a steady, consistent drop in numbers of haemocytes occurred. By 96 hours most injected larvae were dead and haemocyte numbers had dropped to zero though the control was still 1.6×10^6 per ml.

By examining haemolymph the insect response to fungal infection can be assessed. Apart from the production of anti-fungal peptides, like drosomycin, the haemocyte reaction and fungal development within the haemocoel can be ascertained.

Twenty-four hours after injection only two isolates (1007 and 1484) had blastospores in the haemolymph. There were no hyphal bodies or any evidence of the injected conidiospores. After 48 hours two of the injected isolates had produced blastospores in the haemolymph, whilst five had produced hyphal bodies. In two cases both blastospores and hyphal bodies occurred at the same time.

The most probable sequence during fungal infection is for blastospores to be shed into the haemolymph to be followed subsequently by the formation of hyphal bodies and the insect's death by mycosis at 96 hours.

Pendland *et al.* (1993) suggest that blastospores may become opsonised and phagocytosed and could replicate within the haemocytes. This would explain the disappearance of freely circulating hyphal bodies within the haemolymph, yet the reappearance of hyphal bodies at 72 hours and the subsequent death of the insect at 96 hours.

Mazet *et al.* (1994) showed that *B. bassiana* could produce soluble toxic elements which were released into the haemolymph of *Spodoptera exigua* larvae and disrupted haemocyte spreading. In the present experiments, this lack of spreading and loss of filopodia was evident on the *M. sexta* haemocytes and rounding up occurred in most samples from injected insects by 72 hours.

B. bassiana produces a wide range of toxic metabolites and their effect on haemocytes may have an important role in overcoming insect resistance.

Chapter 3

Effect on *Manduca sexta* larvae of *Beauveria bassiana* products.

3 .1 Introduction

A parasite obtains its nutrition at the expense of its host. This may compromise the host, effectively starving it to such an extent that it cannot grow and develop normally.

Entomopathogenic fungi have a range of strategies by which they can obtain host's nutrients. They may produce cuticle-degrading enzymes to facilitate host penetration and acquire nutrients for growth (St Leger, Durrands, Charnley and Cooper 1988b), or as Moore, Reed, Le Patourel, Abraham and Prior (1992) and Seyoum, Moore and Charnley (1994) demonstrated with the desert locust *Schistocerca gregaria,* fungal infection may reduce the level of feeding of the insect. This lack of feeding may be due to an increased glucose concentration in the insect haemolymph during mycosis which depresses appetite and subsequent weight gain.

Deuteromycetes are often confined largely to the haemocoel and prior to the host death benefit from the nutrients contained in the haemolymph. Shimizu, Tsuchitani and Matsumoto (1993) showed that *Beauveria bassiana* produced substantial amounts of extra-cellular proteases in the haemolymph of silkworms and might thus be able to benefit from the breakdown of haemolymph proteins. However, Yoshida, Yamashita, Yonehara and Eguchi (1990) found protease inhibitors in silkworm haemolymph that were capable of inhibiting fungal development so the fungal production of protease within the haemolymph may not always be an effective parasitic strategy. On the insect's death, though, the fungal mycelium may utilise the nutrients contained within the host tissues, in a phase of saprophytic growth.

Although some *B. bassiana* isolates produce mycosis in *M. sexta* larvae, it is likely that fungal metabolites by themselves may have a deleterious effect on the larvae (Kucera and Samsinákóvá 1968). They may be introduced on the surface of the conidiospores, or produced during germination or hyphal growth. If a toxic substance is bound to the surface of the spores heat-treatment could destroy it, so a comparison of heated and unheated spores could indicate whether this is a cause of lack of weight gain.

However, whilst entomopathogenic fungi may restrict larval growth by mycosis the production of toxic metabolites may have a more immediate effect on the host. These

metabolites may be produced at different points of fungal development and may work synergistically, hence even small amounts may reduce larval feeding and weight gain. They may, like destruxins, cause paralysis (Samuels, Reynolds and Charnley, 1988b), or, like oosporein, a change in the insect cuticle colour (Eyal *et al.* 1994).

Although the effects of fungal metabolites are diverse they vary considerably from one insect species to another. Genther, Cripe and Crosby (1994) found that beauvericin, a product of *B. bassiana* , was toxic to mysids, and Hamill, Higgins, Boaz and German (1969) that it was toxic to *Artemia salina*, but Kanaoka, Isogai and Murakoshi (1978) showed that even at high doses it did not affect silk-worm larvae. There are no reports of it being toxic to lepidopteran species.

Boucias and Pendland (1987) showed that *B. bassiana* isolates produced substantial amounts of extra-cellular proteases in the haemolymph of *Anticarsia gemmatalis.* Shimizu *et al.* (1993) showed that the silkworm haemolymph contained protease inhibitors which could prevent the development of entomopathogenic fungi, however, although proteases might be produced, it is not certain that they are active *in vivo.* The ability of insects to inhibit fungal enzymes may vary according to the host species and hence the effect of *B. bassiana* on *M. sexta* may not be the same as on *Bombyx mori.* There may also be variation between fungal isolates and it is possible that some *B. bassiana* isolates vary in their ability to produce proteases which *M. sexta* larvae could not inhibit and which would reduce the insect's ability to gain weight.

Chen *et al* (1999) isolated a β-1,3 glucan-specific lectin from the serum of the cockroach, *Blaberus discoidalis*, which interacted with insect haemocytes. Lectins have been shown to elicit host defence mechanisms against fungal infection and to mediate cell-based immunity (phagocytosis and haemocyte aggregation) in both horseshoe crabs and in the insect *Manduca sexta.* The β-1,3 glucan-binding protein which was identified was also shown to act as an opsonin in infections of the cockroach (Wilson, Chen and Ratcliffe, 1999).

If the fungus produces metabolites which are toxic to the insect, the ability of the host to combat infection will be reduced. Boucias *et al.* (1994) showed that *Beauveria bassiana* could disrupt the immune system of the beet army worm, *Spodoptera exigua.* The hyphal bodies of the fungus within the haemolymph appeared to be coated with host proteins and so avoided phagocytic recognition, so the insect was unable to combat infection. The result of this fungal strategy is two-fold, the fungus is able to

develop more quickly with a ready source of nutrients and the insect's ability to grow is reduced.

Other adaptations in the host-parasite competition relate to immune defences. While the insect host has acquired the ability to direct cellular defences against certain invading fungal parasites, the fungus has also acquired the ability to evade or disable the immune responses of certain insects. This is almost certainly an important factor in determining host range. In the present context, it may be that some of these immune phenomena affect nutrient flow and lead to poor growth in the host even when the fungus is ultimately unsuccessful in causing disease. Of particular importance is the production of fungal metabolites during infection. Some of these metabolites may impair insect growth, either through their frank toxicity, or by affecting the insect's feeding behaviour.

Different *B. bassiana* isolates produce a wide range of metabolites. However, there exist few records of the sub-lethal effects of these toxins on Lepidoptera. A large number of *B. bassiana* isolates produce both beauvericin and bassiatin in liquid culture (Kagamizono and Nishino 1995) Beauvericin is a depsipeptide of known structure with deleterious effects against *Mysidopsis bahia* (Genthner *et al.* 1994), whilst bassiatin has been shown to have inhibitory activities against rabbit platelet aggregation. Neither of these two metabolites has been tested against *M. sexta.*

Bassianolide, a depsipeptide produced by *B. bassiana* has been shown to be toxic to *Bombyx mori* (Murakoshi, Ichinoe, Suzuki, Kanaoka, Isogai and Tamura 1978) and, like destruxin, produced by *Metarhizium anisopliae*, may cause temporary paralysis in larvae. Destruxin inhibits insect haemocyte function and, in vitro, the production of ecdysterone by the prothoracic glands of *Manduca sexta* larvae (Sloman and Reynolds 1993). Bassianolide has a similar structure to the cyclic peptide destruxin and it is conceivable that it has similar effects and could interfere with the onset of pupation. Since *M. sexta* larvae are unable to pupate until they have reached a certain body weight, normally about 8 grams, retardation of pupation could be due merely to low larval weight, but bassianolide might also affect the production of insect hormones. Whilst *B. bassiana* is able to produce a large number of metabolites, some of these metabolites may have little effect on *M. sexta* larvae. Others, singly or synergistically, may reduce weight gain or cause malaise.

The investigation of the pathogenicity of *Beauveria bassiana* to *Manduca sexta* larvae (Ch. 2) suggested that insect deaths might not be entirely due to mycosis. The lack

of weight gain and malaise exhibited by some larvae after treatment with the fungus may be due to fungal metabolites, either on the surface of germinating spores, or produced as the fungus penetrates the cuticle and gains entry to the haemolymph.

By using sterile filtrate from *B. bassiana* liquid cultures the effect of fungal growth on the insect was avoided and, although the interaction between fungus and insect cannot be shown as it might be *in vivo,* the effect of fungal metabolites on the insect becomes clearer.

B. bassiana has been known as an insect pathogen for over 200 years and there exist a large number of isolates with varying ability to affect different insect species. It may be possible to show that isolates vary in their ability to produce metabolites which affect *M. sexta* larvae.

3.2 Materials and methods

3.2.1 Insect culture and fungal spore culture was the same as in 2.2.1 and 2.2.2. Insect injections were the same as 2.2.3.

3.2.2 Use of heated spore suspensions

Five ml aliquots of 2 × 10^6 spores per ml of each isolate (suspended in distilled water) were placed in glass universal bottles and autoclaved at 120° C for 20 mins. They were then cooled to 20° C and shaken on a vortex mixer for 30 s. Ten microlitres of these suspensions was injected into 10 newly ecdysed 5th instar *Manduca sexta* larvae which were then incubated at 25° C , 17/7 hr light/dark and 45% humidity for 5 days. Five samples of ten microlitres of each suspension was also plated on SDAY and incubated at 25° C in the dark, together with unheated spore suspensions as a control.

3.2.3 Preparation of fungal filtrates

a). Growth

Cultures of all 12 isolates were streaked from stock plates onto SDAY and grown and harvested as before. Spore suspensions in sterile distilled water were counted on a Neubauer haemocytometer and adjusted to 1 × 10^7 spores per ml. One hundred ml of Czapek Dox or Sabouraud Dextrose liquid media (see Appendix) were placed in 250 ml conical flasks.

The liquid media were inoculated with 1 ml of spore suspension at 1 × 10^7 spores per ml and then incubated at 25° C on a Gallenkampf orbital shaker at 120 rpm with continuous light for 8 days. Two ml was removed from each flask at 24 hour intervals and the pH tested.

After 8 days, samples of each culture were checked microscopically for growth development and contamination.

b). Filtration

The liquid 8-day cultures were filtered through a double layer of sterile muslin. The filtrate was then centrifuged at 1407 *g* for 20 min, the supernatant decanted and centrifuged again at 1407 *g* for 20 minutes .

The supernatant was then held on ice and 10 ml aliquots filtered through 0.45 µm Acrodiscs (Gelman Sciences) to remove any remaining spores or hyphal fragments. Controls of un-inoculated C-Dox liquid media and SDL were also filtered through Acrodiscs and all samples were stored at 4° C for 48 hours prior to injection.

The mycelia from each culture were placed in pre-weighed aluminium boats and dried in an oven at 65° C until a constant weight was obtained.

Fungal filtrate preparation and weighing of dried mycelia was repeated twice (three replicates).

Two SDAY plates were inoculated with 50µl of filtrate from each isolate and incubated at 25° C for 7 days to check that the filtrate did not contain any live fungal material.

c). Injection

Culture filtrates were allowed to reach ambient temperature (22° C) prior to injection. Newly ecdysed 5th instar *M. sexta* larvae (5 per treatment) were weighed and were then injected with filtrate into the inter-segmental membrane between the seventh and eighth segment using a 100µl Hamilton syringe directed anteriorally. The syringe needle was rinsed with 70% ethanol between injections to prevent cross-infection between insects and then 3 times with sterile distilled water between different treatments and isolates.

Culture filtrate from each isolate grown in SDL or C-Dox liquid was injected at 4 different doses, 10 µl, 30 µl, 50 µl and 70 µl. Controls were injected with similar doses of sterile SDL or C-Dox liquid.

After injection, the insects were returned to individual pots containing Manduca diet (see Appendix) and incubated at 25° C with 45% RH and a 17/7hr light/dark regime. They were weighed at 24 hour intervals and at 5 days haemolymph was examined from an insect treated with each isolate.

3.2.4 The production of oxalic acid in *Beauveria bassiana* culture filtrates and its effect on *Manduca sexta* larvae

There are several records of the identification of oxalic acid in fungal filtrates (Godoy, Steadman, Dickman and Dam 1990; Espejo and Agosin 1991; Bidochka and Khachatourians 1993). However, although different workers have considered the effects of the acid on the exterior of both plants and insects, the effects within the living organism do not appear to have been investigated.

Bidochka and Khachatourians (1991) considered the solubilisation of insect cuticle by oxalic acid and tested strains of *Beauveria bassiana* against *Melanopus sanguinipes.* They concluded that the hyper-production of oxalic acid was not linked to the virulence of the fungus against the insect.

The production of oxalic acid by *B. bassiana* may vary between isolates, but its effects on *M. sexta* larvae are not recorded.

Culture filtrates from the growth in liquid media of the twelve different isolates were compared with known solutions of oxalic acid for their effect on larval weight gain. The effect of the acid on the larval haemolymph was also investigated.

The method of fungal culture in liquid media was as described in 3.2.3. Czapek-Dox was used as the liquid medium. Prior to use, 10 ml aliquots of the fungal filtrate were thawed and passed through a Gelman Acrodisc 0.2/0.8 μm (Gelman Sciences, Northampton, U.K.).

3.2.5 Determination of oxalate in the culture filtrate samples

Each of the twelve fungal filtrates was assayed for oxalic acid using a Sigma (Poole) diagnostic kit. The test depends on an enzymatic reaction and the production of colour from a dye. The intensity of colour is directly proportional to the amount of oxalate present.

The oxalate is converted by oxalate oxidase into hydrogen peroxide and carbon dioxide. The peroxide then reacts with 3-methyl-2-benzothiazolinone hydrazone (MBTH) and 3-(dimethylamine)benzoic acid (DMAB) to yield an indamine blue and water. The intensity of colour is read at 590 nm and from these results the concentration of oxalic acid in the sample can be calculated.

1) $$\text{Oxalate/oxalic acid} + O_2 \xrightarrow{\text{Oxalate oxidase}} 2CO_2 + H_2O_2$$

2) $$H_2O_2 + \text{MBTH} + \text{DMAB} \xrightarrow[\text{peroxidase}]{} \text{Indamine dye} + H_2O$$

The Sigma (Poole) procedure was followed as detailed below.

1) The culture filtrate samples were thawed and allowed to reach 22° C. They were then passed through an Acrodisc (Gelman Sciences) with 0.8/0.2 μm pore size.

2) 3 ml of each culture filtrate and 3 ml of freshly made sample diluent (EDTA 10m.mol/litre, buffered to pH 7.6 ± 0.1 and dissolved in distilled water) was added to each tube.

3) The pH was adjusted to between pH 5 and pH 7 with 1N HCl or 1N NaOH

4) Standards were made up of 400, 200, 100, 50 and 25 mg of oxalic acid each dissolved in a litre of distilled water.

5) 1 ml of oxalate Reagent A was added to each of a series of empty tubes. This proprietary reagent contains DMAB and MBTH in a buffered solution at pH 3.1.

6) 50 μl of standard, culture filtrate, C-Dox (control) or distilled water (blank) was added to each tube.

7) 0.1 ml of Reagent B (oxalate oxidase and horseradish peroxidase) was added to each tube and mixed by inversion.

8) Samples were transferred to a 96-well flat-bottom microtest plate (Stratagene, Amsterdam) and incubated at 22° C ± 1° C for 5 min. The absorbance was then read at 590 nm on a Dynatech MYR 5000 plate reader.

9) A calibration curve was prepared and the concentration of oxalic acid in filtrate samples determined by subtracting the reading for the blank, comparing with the calibration curve and correcting for sample dilution.

Presence of catalase

Since the enzymatic test for oxalic acid depended on the production of hydrogen peroxide and its reaction with MBTH and DMAB, each filtrate was tested for the presence of hydrogen peroxide with a solution of catalase.

Fifty microlitres of 0.25% freshly made up catalase solution (Sigma) was added to 50 μl of each filtrate in an assay plate at 22° C. A control was established with 50 μl

of catalase solution and 50 µl of 35% hydrogen peroxide (Aldrich Chemicals). The plates were observed for 4 min. after which time the control plates no longer reacted.

Oxalic acid in Czapek-Dox (control)

Three samples of sterile liquid Czapek-Dox, the medium in which the fungus had been grown, were tested for the presence of oxalic acid.

3.2.6 Comparison of weight gain of larvae after injection with oxalic acid, or culture filtrate from isolate 2727

Five fifth instar *M. sexta* larvae were injected longitudinally with 50 µl of culture filtrate from isolate 2727 through the inter-segmental membrane between the seventh and eighth segments. The insects were incubated at 24° C ± 1° C, 45% RH and a 17/7 h light/dark regime for 5 days. Each insect was weighed at 24 h intervals and the onset of "wandering" (pre-pupation behaviour) noted.

A similar bioassay was carried out injecting larvae with different dilutions of oxalic acid. Six different dilutions, from 100 to 1000 µg/l of oxalic acid in sterile liquid Czapek-Dox were used, with fifty microlitres of sterile C-Dox for control insects. Larval weight gains were compared at 24 hour intervals over 5 days.

The bioassays were replicated three times using five insects for each dilution or isolate filtrate.

3.2.7 The oxalic acid content of infected larval haemolymph

The oxalic acid content of first day fifth instar (1d5L) larval haemolymph was compared after injection with isolate 2727 spores and after injection with commercial oxalic acid.

The enzyme assay and standards previously developed were used.

a) Treatment with fungal spores

Groups of five fifth instar larvae were injected with 50 µl of spores of isolate 2727 at a concentration of 9.2×10^6 spores per ml (as in 2.2.3). The larvae were incubated at 24° C ± 1° C, 45% RH and 17/7 h light/dark regime.

b) Oxalic acid injection

A solution was made of 2g of oxalic acid in 100 ml of sterile Grace's Insect Medium and balanced to pH 6.5 with 1N NaOH. The solution was filtered through a

0.8/0.2 μm Acrodisc (Gelman Sciences) and injected at the rate of 50 μl per larva, hence 1000 μg (1 mg) of oxalic acid per insect.

The larvae were incubated as in a).

c) Haemolymph collection and treatment

After incubation the larvae were placed in pots on ice for 30 min. They were then wiped, first with sterile distilled water, and then with 70% alcohol. The insect horn was removed with flamed scissors and the haemolymph collected in sterile microcentrifuge tubes containing a few crystals of phenylthiourea (PTU) to prevent melanisation.

Haemolymph from each group of five larvae was pooled and then tested for the presence of oxalic acid.

d) Spiked haemolymph

Three haemolymph samples (each of 12.5 μl) from non-treated larvae were spiked with 12.5 μl of a solution of 20g/l of oxalic acid, giving a final concentration of 10 μg/μl. They were compared with the oxalic acid content of non-treated haemolymph samples.

3.2.8 The effect of pigments produced by *B. bassiana* on *M. sexta* larvae

Twenty first day fifth instar larvae were each injected with fifty microlitres of conidial suspension ($1x10^6$ spores per ml) from the *Beauveria bassiana* isolates producing pink colouration during growth on SDA (Isolates 1122, 1315, 1484, 1629, 2417 and 2727).

A further twenty, first day fifth instar larvae were immersed for ten seconds in conidial suspensions of the same isolates. Treatment with isolate 304, which did not produce pink pigmentation on SDA, was used as a control.

The larvae were incubated at 25°C ± 1°C and 17/7h light/dark for 7 days. They were then examined for the presence of pnk pigmentation.

3.3 Results

3.3.1 Injection of heat-treated spores

Larvae inoculated with heat-treated spores gained weight at a similar rate to control larvae which had been injected with sterile distilled water (see Table 9b). By contrast, larvae inoculated with the same concentration of unheated spores (10µl of 3 x 10^6 spores per ml) from the same isolates, significantly ($p< 0.05$) failed to gain weight by 24 hours.

The difference in larval weight gain between the injection of live and dead spores suggested that if an agent existed on the outside of the spores which could affect insect weight gain the heat-treatment had destroyed it.

There was no fungal or microbial growth on the SDAY plates which had been inoculated with heat-treated spores after 7 days incubation so the heat-treatment was assumed to be effective in rendering spores non-viable. Normal growth occurred on the plates inoculated with untreated spores.

Figures 7 and 8 for isolates 1315 and 1484 are typical of all the isolates and show that weight gain for the control insects and those treated with heated spores both have upward trends.

Analysis of variance (ANOVA) showed no significant difference between the weight gain of the control larvae (injected with sterile water)and those injected with heat-treated spores. There was a significant difference ($p<0.01$) from 24 hours between the weight gain of insects treated with heated, or unheated conidiospores.

Table 9 (b) Effect of injection of heat-treated conidiospores (10µl of 1x 10^6 spores per ml) of different isolates of *B. bassiana* on weight gain of *M. sexta* 1d5L larvae.

Isolate number ↓	Weight gain (in grams) after incubation (hours)			
	24h	48h	72h	96h
304 heated	0.72g	1.85g	3.695g	4.67g
non-heated	-0.1	-0.1	-0.3	-1.38
959 heated	0.9	2.607	4.83	7.48
non-heated	0.647	2.133	3.153	4.47
1007 heated	0.90	2.45	4.83	7.50
non-heated	0.906	1.06	1.257	1.24
1122 heated	0.68	2.29	4.38	7.46
non-heated	0.33	0.23	0.32	0.37
1315 heated	0.82	1.8	3.75	5.65
non-heated	0.25	0.31	0.46	0.27
1484 heated	1.03	3.33	5.25	7.42
non-heated	1.03	1.01	1.00	0.95
1558 heated	1.24	3.12	5.12	7.51
non-heated	0.81	1.96	2.75	3.74
1629 heated	1.22	3.52	5.56	7.15
non-heated	0.56	0.80	0.70	0.57
1886 heated	1.01	2.91	5.57	7.12
non-heated	0.48	0.40	0.41	0.77
2417 heated	1.12	2.95	5.35	7.15
non-heated	0.64	1.01	1.55	3.18
2727 heated	1.01	2.91	4.79	7.50
non-heated	0.62	0.9	1.29	1.02
3527 heated	0.81	2.37	4.15	7.17
non-heated	1.21	1.26	1.21	0.72
Control (no spores)	1.05	2.87	5.52	7.91

n = 15 (5 larvae x 3 replicates)

Figures 7 and 8

Figures 7 and 8 show the effect on larval weight gain of injecting heat-treated conidiospores of isolates 1315 and 1484 into first day fifth instar larvae.

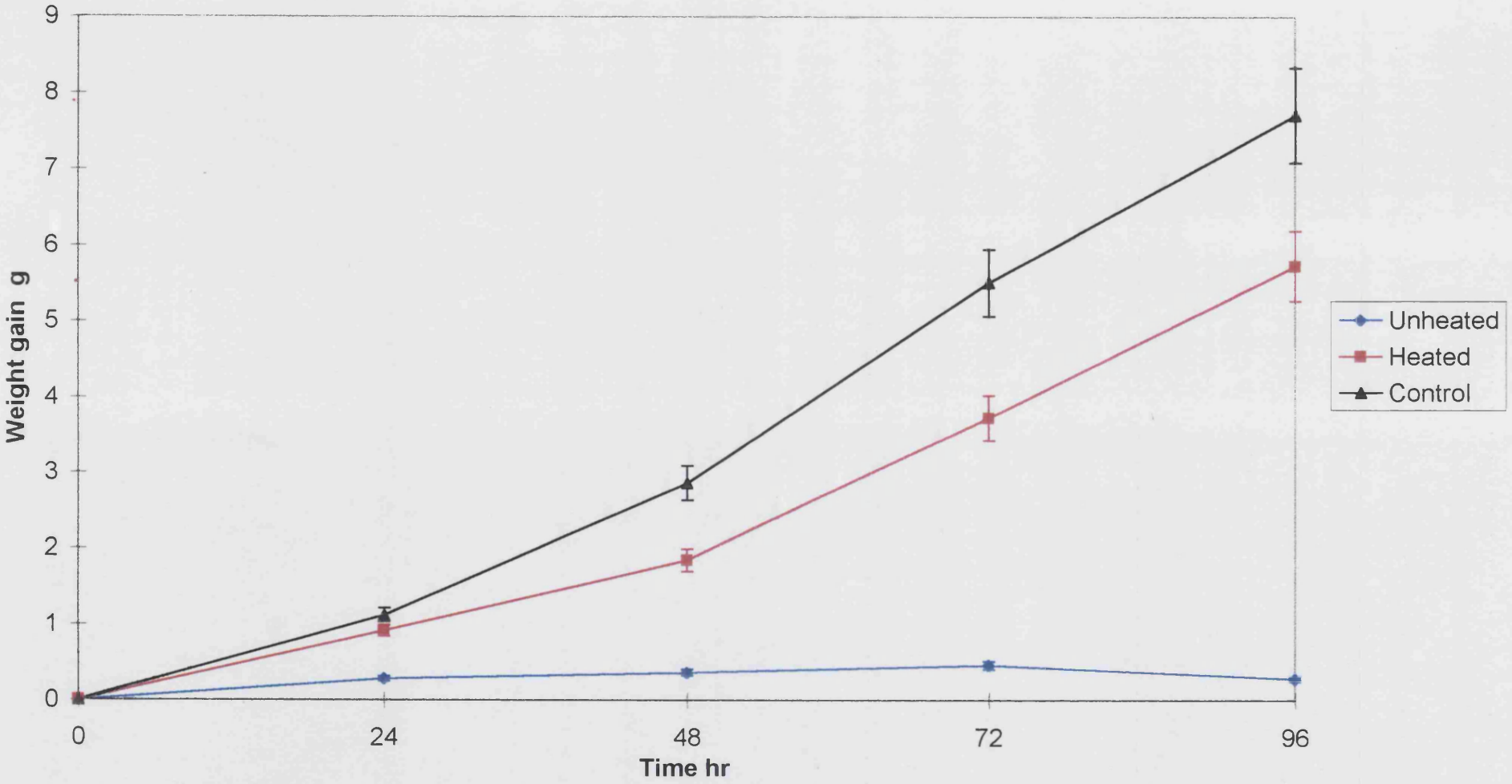
Spores of these two isolates were harvested with 0.05% Tween after 8 days growth on SDAY. The conidiospores were washed and suspended in distilled water and the concentration adjusted to 3×10^6 spores per ml. Five ml aliquots of spore suspensions from both isolates were heated to 120°C for 20 minutes. After cooling to 20°C the suspensions were shaken for 30 seconds on a vortex mixer and 10µl injected into each of ten larvae. The larvae were incubated at 25°C ± 1°C for 96 hours and weighed at 24 hour intervals.

All twelve isolates of *Beauveria bassiana* were tested in this way, with unheated spore suspensions of each isolate as controls.

The work was repeated 3 times (n = 30)

Analysis of variance on the weight gain of larvae injected with unheated spores of isolates 1315 and 1484 showed a significant difference from the control ($p<0.01$). This difference was evident at 24 hours for isolate 1315 and 48 hours for isolate 1484. There was no significant difference in weight gain between larvae injected with heated spores of isolates 1315 and 1484 and the control.

FIGURE 7. Effect of heat-treated spores on larval growth rate
Isolate 1315

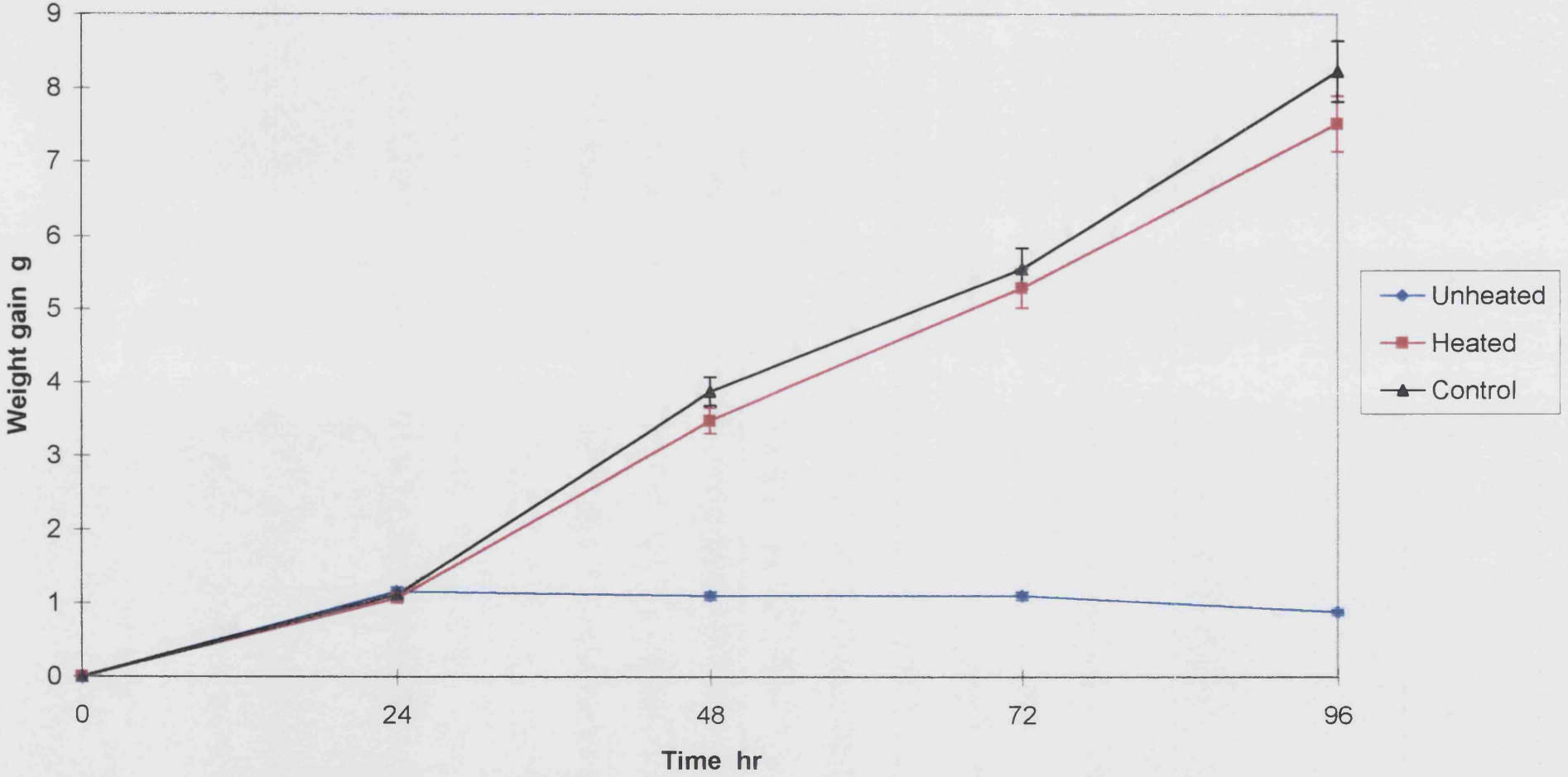


of 3 x 10^6 spores
per ml

Injection of 10 µl

n = 10

FIGURE 8. Effect of heat-treated spores on larval growth rate Isolate 1484



Injection of 10µl of 3 x 10^6 per ml heated and unheated spores.
n = 10

3.3.2 Change in pH of liquid cultures

All twelve isolates showed a drop in pH which reached low points between pH 3 and pH 5 after 48 hours (see Table 10). Although there was some variation between the isolates, the general pattern was a fairly steep drop followed by a slow recovery to between pH 4 and pH 5.5 at 168 hours.

Table 10. Changes in pH of liquid medium during growth of *B. bassiana* isolates.

Isolate	24 h	48 h	72 h	96 h	168 h
304	5.85	4.80	4.38	4.43	4.25
959	6.49	4.86	4.74	4.66	4.84
1007	5.85	4.19	4.29	4.65	5.69
1122	6.48	4.63	3.48	3.58	4.25
1315	6.22	3.31	4.69	4.96	4.57
1484	6.37	4.00	3.77	4.03	5.27
1558	5.62	3.86	4.36	3.55	4.28
1629	5.09	3.52	4.10	3.94	4.02
1886	5.83	3.51	3.99	4.80	6.10
2417	6.38	4.21	4.08	4.27	5.71
2727	5.30	3.51	4.28	4.88	5.59
3527	5.34	3.45	4.45	3.94	5.28
Control	6.88	6.81	6.90	5.83	6.72

One ml of spore suspensions of each isolate at 1×10^7 was inoculated into flasks containing 100 ml of Czapek-Dox liquid media. The cultures were incubated on an orbital shaker at 120 rpm in continuous light for 8 days.

Two ml was removed from each culture at 24 hour intervals and the pH tested. The work was repeated 3 times.

3.3.3 Variation in mycelial weight between isolates

The amount of mycelia produced during growth in liquid culture may influence the quantity of metabolites released into the filtrate. To monitor this the mycelia retained after filtering the cultures were placed in pre-weighed aluminium boats and dried at 65° C until a constant weight was obtained (96 h).

The mean weights of the mycelia from the different isolates, together with their ranking in weight are given in Table 11, with 1007 the heaviest and 1629 the lightest.

Table 11.

Dried mycelial weight from 8-day liquid cultures of different isolates.

Isolate	Mean mycelial dry weight g.	Ranking	S.E.M.
304	1.206	8	0.1280
959	1.6595	3	0.1730
1007	2.0397	1	0.4091
1122	0.9937	9	0.0295
1315	1.937	2	0.3559
1484	1.5096	5	0.1572
1558	1.5754	4	0.3711
1629	0.9179	12	0.0602
1886	1.474	6	0.0717
2417	1.447	7	0.1939
2727	0.9759	10	0.0885
3527	0.9652	11	0.0486

(n=3)

The cultures were grown in Czapek-Dox liquid media for 8 days (as for Table 10). The mycelia was collected and dried at 65°C to a constant weight and the work repeated 3 times.

The mean dry weight of the mycelia from all 12 isolates was 1.3464 g with a deviation between the isolates of 0.343 g. Analysis of variance showed that there was no significant difference at the 5% level of probability between the dry weights of mycelia of different isolates.

3.3.4 Injection of mycelial filtrates

None of the 24 SDAY plates (two for each isolate) which had been inoculated with culture filtrate and incubated at 25° C had any microbial growth after 7 days, so the culture filtrates were assumed to be sterile.

Larval weight gain was used to measure the effects of filtrate inoculation.

a) Comparison of the effects on larval growth of culture filtrate from mycelia of four different isolates grown in different media(see Table 12 overleaf)

A dose of 50µl of culture filtrate from isolates 959, 1315, 1886, and 2727 grown in either Czapek-Dox or SDL was inoculated into each insect. Five insects were used for each dose and the trial repeated three times (a total of 15 insects for each treatment). Analysis of variance showed that there was no significant ($p<0.05$) difference in larval weight gain between filtrates grown in the two media.

Sterile liquid Czapek-Dox, SDL and GIM were used to inject the control insects. Czapek-Dox media has a high salt concentration which could have been mildly toxic to the larvae whilst SDL, with 40 g per litre of dextrose, could affect the osmotic balance within the insect.

The controls, where larval weight gain after injection with the two different sterile media and GIM were compared and showed that the media did not significantly ($p> 0.05$) affect the insect weight gain. The insect larvae appeared to tolerate these levels of media salts.

Table 12.

Average total weight gain (g) of larvae 96 hr after injection with culture filtrates from different isolates grown in C-Dox liquid and SDL

Isolate	Czapek-Dox		SDL		GIM	
—	Mean	SEM	Mean	SEM	Mean	SEM
959	6.77	0.35	7.18	0.02		
1315	1.33	0.12	0.89	0.32		
1886	3.75	0.29	3.78	0.18		
2727	4.43	0.12	4.20	0.08		
Control	6.6	0.17	6.30	0.12	7.15	0.08

n = 15

First day fifth instar larvae were injected with 50µl of fungal filtrates from four different isolates grown in either C-Dox liquid, or SDL. The larvae were incubated at 24°C and weighed at 24 hour intervals and their total weight gain at 24 hours compared.

The weight gains of larvae treated with culture filtrate from isolates grown in the two media, Czapek-Dox and SDL were not significantly different($p>0.05$). However, analysis of variance showed that weight gain at 96 hours of larvae injected with culture filtrates from isolates 1315 and 1886 was significantly ($p<0.05$) different from the control.

b) Comparison of the culture filtrates from 12 different isolates on larval growth

Since it has been shown (above) that filtrate from cultures grown in C-Dox and SDL gave similar results on insect weight gain, filtrate from 12 different isolates grown in C-Dox was compared.

M. sexta larvae were injected with 50µl of culture filtrate from all 12 isolates which had been grown in C-Dox. A significant ($p<0.05$) difference occurred in the rate of weight gain between the larvae treated with different isolates.

No culture filtrate caused a knockdown effect and none caused paralysis at this relatively small dose of 50µl.

Ranking the weight gain (Table 13, overleaf) gave an indication of the most effective isolates.

Table 13.

Ranked 96 hr weight gain of newly ecdysed 5th instar larvae injected with 50µl of culture filtrate from 12 *B. bassiana* isolates

Rank	Isolate	Weight gain (g)	S.E.M.
1	1886	8.40	0.36
2	1484	8.1	0.56
3	2417	7.94	0.24
4	Control	7.66	0.43
5	1629	7.42	0.33
6	959	6.94	0.29
7	1122	6.72	1.12
8	1007	6.22	0.81
9	304	5.80	1.17
10	1315	5.60	0.35
11	2727	5.40*	0.48
12	1558	3.92*	0.82
13	3527	3.86*	0.51

(3 insects in each of 3 replicated trials n = 9)

Fifty microlitres of culture filtrates from all twelve isolates was injected into first day fifth instar larvae which were incubated at 25°C and weighed daily. Their weight gain at 96 hours was ranked. Stars (*) indicate culture filtrates which significantly ($p<0.05$) reduced larval weight gain.

Of the isolate filtrates (Figures 9,10 and 11 as summarised in Table 13) which affected weight gain some like 2727, 3527 and 1315 had a continuous effect whilst others like 1558 and 304 had a greater effect after 72 hours. There were no larval deaths at 96 hrs.

Figures 9, 10 and 11

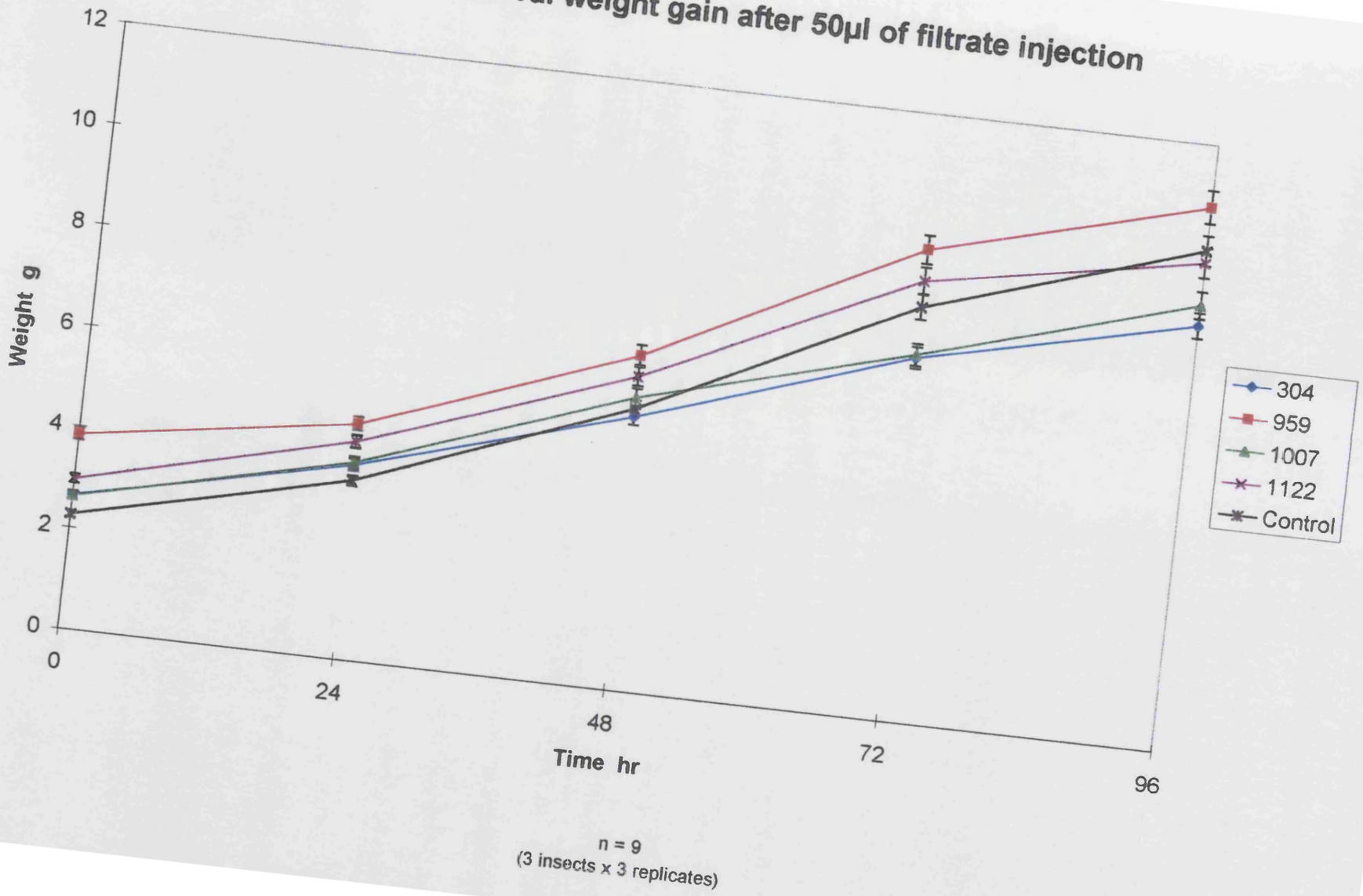
Figures 9, 10 and 11 show the effect on larval weight gain from 0 to 96 hoursof the injection of 50µl of fungal filtrate from the *in vitro* culture of twelve different isolates of *Beauveria bassiana* (as in Table 13, page 85).

One ml of conidiospores of 1×10^7 spores per ml suspension was added to 100 ml Sabouraud Dextrose liquid media in 250 ml conical flasks. The cultures were incubated at 25°C on an orbital shaker at 120 rpm, with continuous light, for 8 days. They were then filtered through sterile muslin and the filtrate centrifuged at 1407*g* for 20 minutes. Ten ml aliquots were then re-filtered through 0.45µm Acrodics (Gelman Sciences) to remove any remaining spores or hyphal fragments.

All the isolates were tested by injecting 50µl of fungal filtrate into first day fifth instar larvae (3 per isolate), which were then incubated at 25°C ± 1°C and weighed at 24 hour intervals.

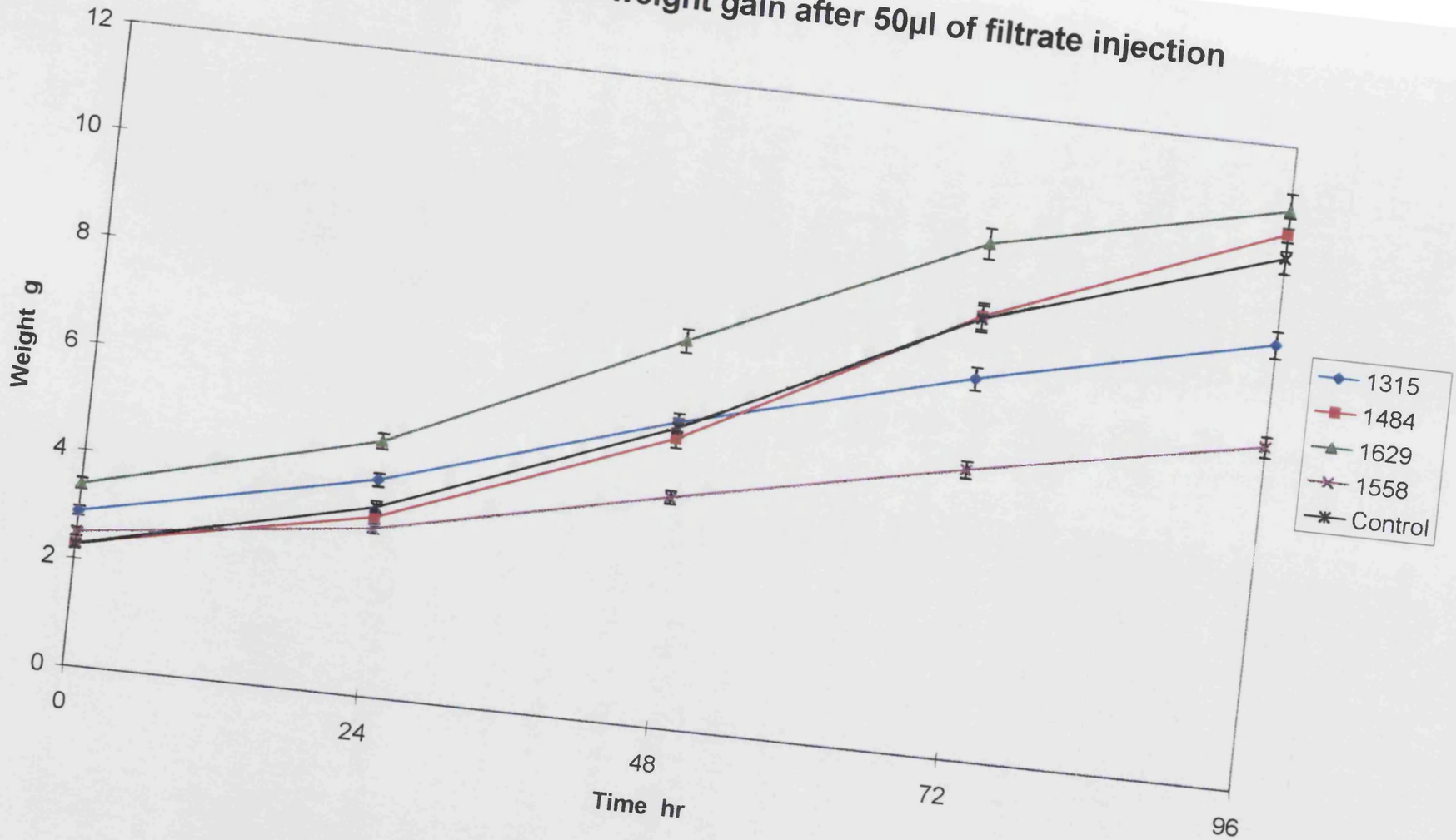
The work was repeated 3 times (n = 9).

FIGURE 9. Larval weight gain after 50µl of filtrate injection



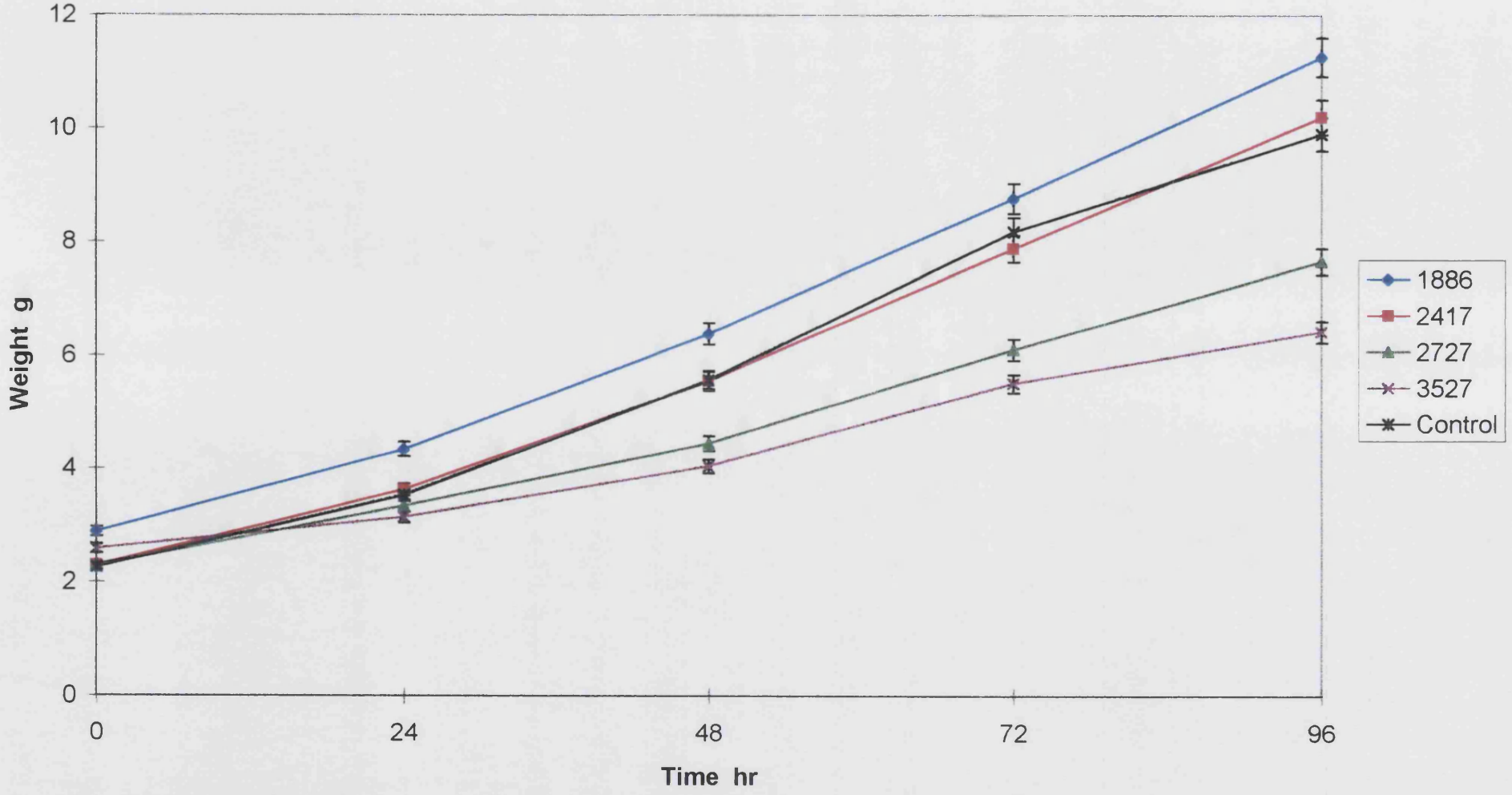
n = 9
(3 insects x 3 replicates)

FIGURE 10. Larval weight gain after 50µl of filtrate injection



n = 9
(3 insects x 3 replicates)

FIGURE 11. Larval weight gain after 50µl of filtrate injection



n = 9
(3 insects x 3 replicates)

c). Effect of culture filtrate dose on larval weight gain

Four doses, 10µl, 30µl, 50µl and 70µl of culture filtrate were used with 3 insects per dose and 3 replicate trials for each isolate. Most of the newly ecdysed 5th instar *M. sexta* larvae weigh between 1.5 and 2 g and injection above 100µl, i.e. more than 5% of the body weight, could induce stress factors which might influence results.

It was evident from the results that the filtrate from isolates 959 and1886 (Figures 12 and 14) had the least effect on larval weight gain. Analysis of variance showed that culture filtrate from these two isolates did not have a significant effect on larval weight gain at any dose level used. By contrast, injection of only ten microlitres of culture filtrate from isolate 1315 (Figure 13) gave a significant ($p>0.05$) reduction in weight gain after 24 hours and at 96 hours the significance was $p>0.01$.

Injection of ten microlitres of culture filtrate from isolate 2727 gave no significant difference at 96 hours, but an increased dose of seventy microlitres caused a significant ($p>0.02$) reduction in weight gain compared to the control from 48 hours onwards.

There were no larval deaths from the control insects inoculated with sterile media.

Figures 12 , 13 , 14 and 15

These figures show the effect on weight gain of injecting fifth instar larvae with different doses of culture filtrate from isolates of *Beauveria bassiana* (959, 1315, 1886 and 2727).

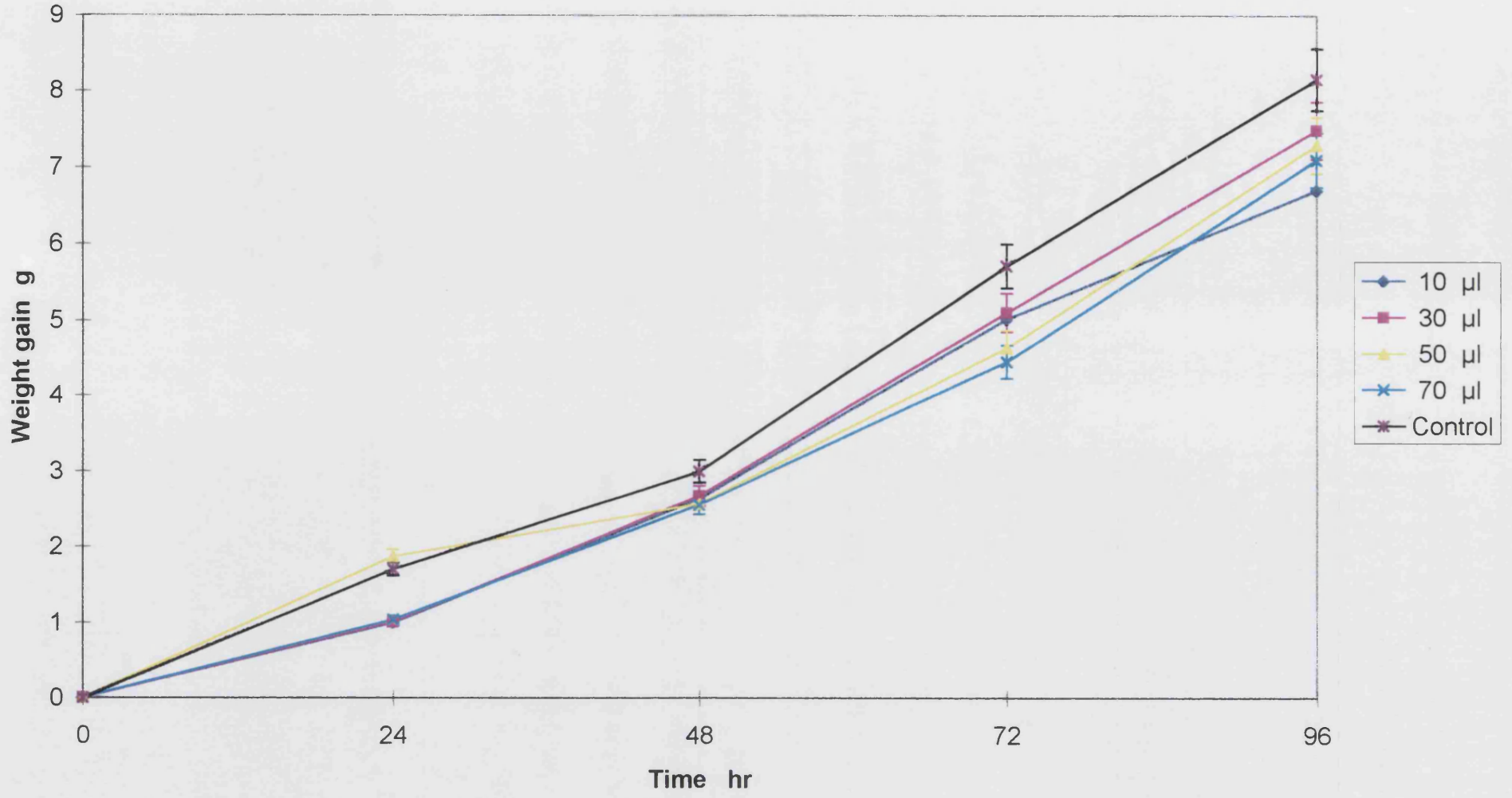
One ml of spore suspension of 1×10^7 spores per ml was inoculated into 100 ml of liquid Czapek-Dox media in 250 ml conical flasks. The cultures were incubated at 25°C on an orbital shaker at 120 rpm with continuous light.

After 8 days the cultures were filtered through sterile muslin and the filtrate centrifuged at 1407*g* for 20 minutes. The supernatant was re-centrifuged and 10ml aliquots were filtered through 0.45 µm Acrodiscs (Gelman Sciences) to remove any remaining spores or hyphal fragments.

Doses of 10, 30, 50, and 70µl of fungal filtrate were injected into first day fifth instar larvae with 3 insects being used for each dose. The work was repeated 3 times (n = 9). Controls were set up using the same doses of sterile liquid Czapek-Dox.

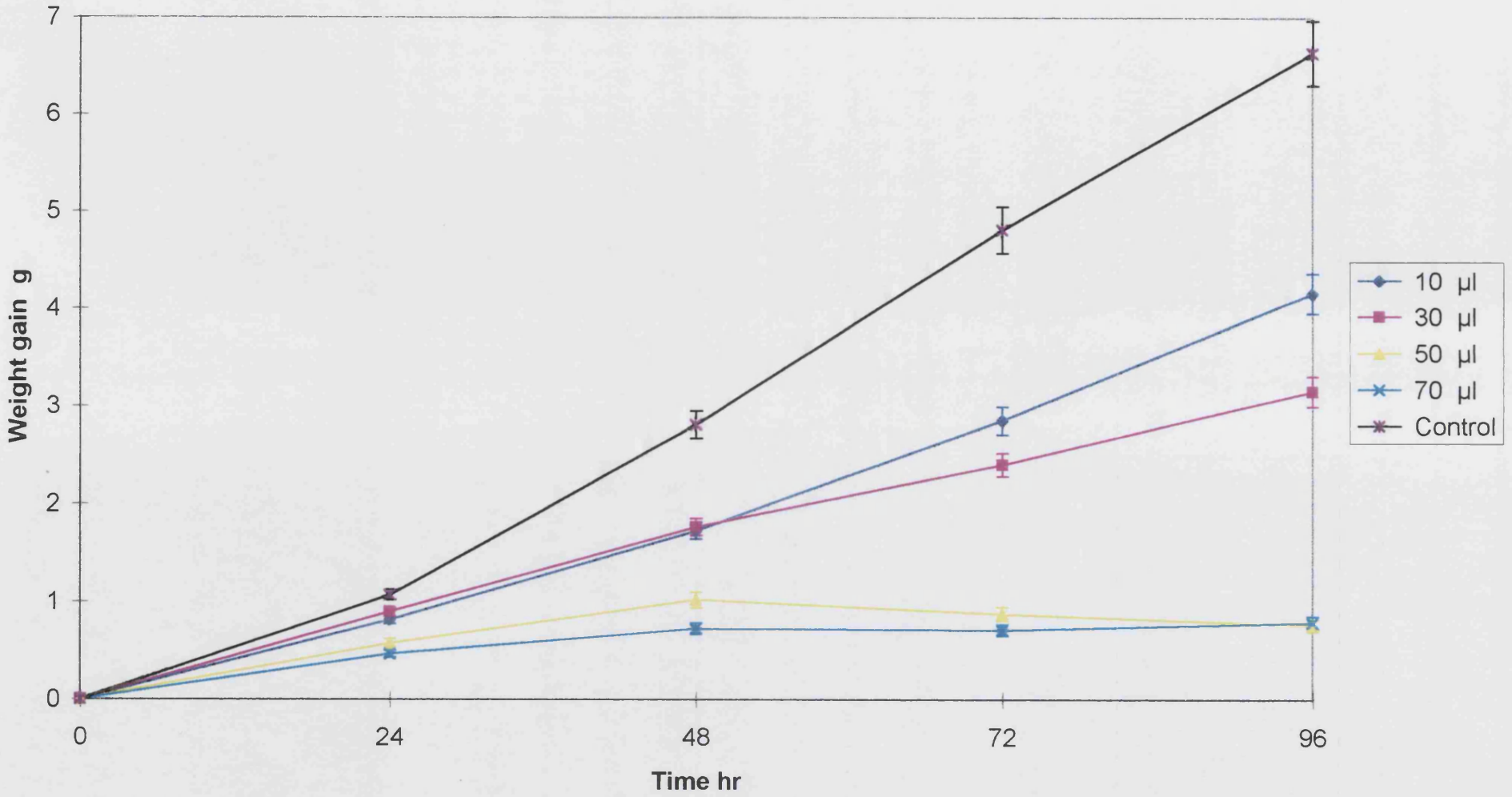
The larvae were incubated at 25 °C ± 1°C with a 17/7 hour light/dark regime and weighed at 24 hour intervals.

FIGURE 12. Effect of 959 filtrate dose on larval weight gain



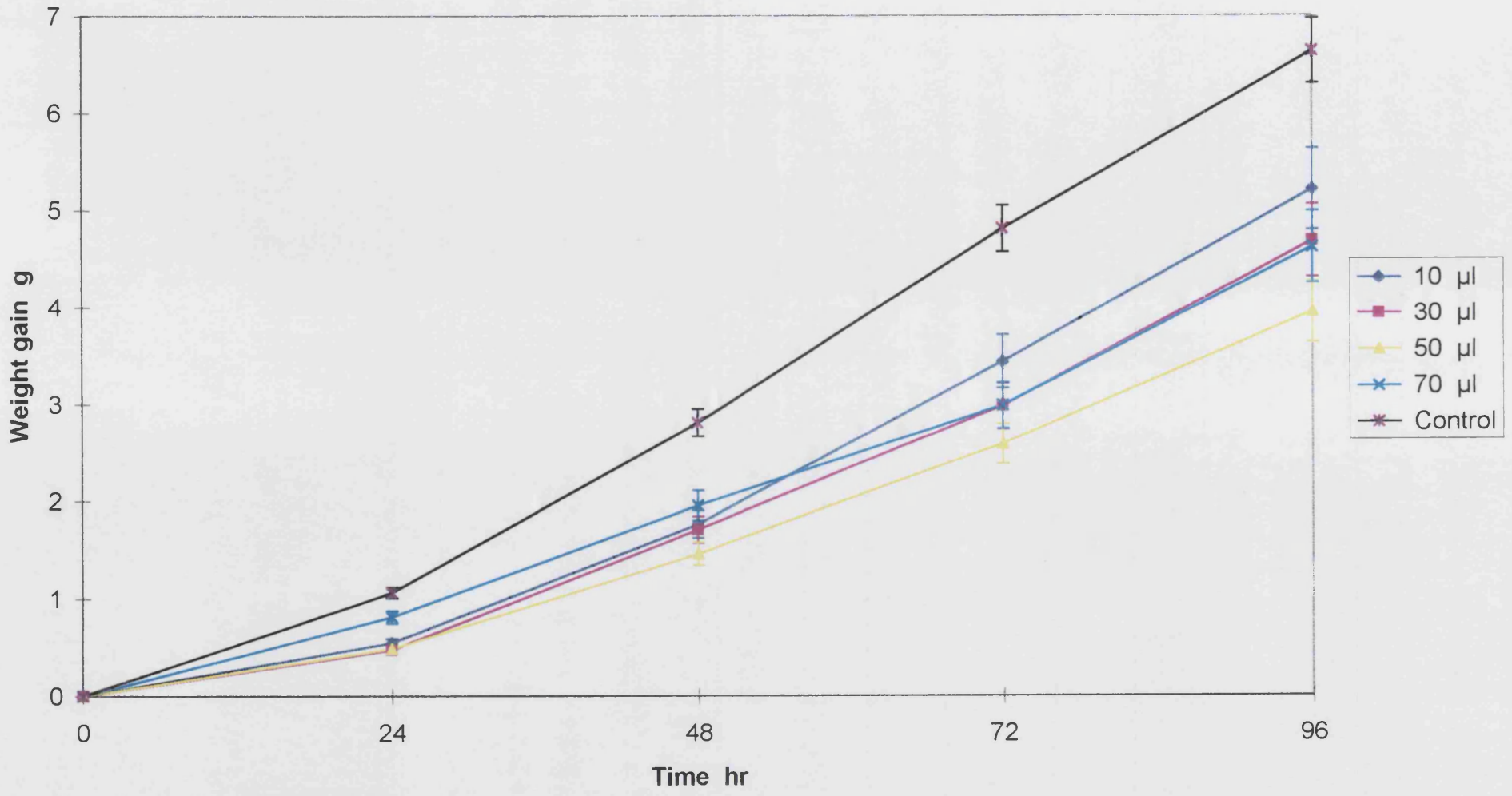
(n = 9 per dose)

FIGURE 13. Effect of 1315 filtrate dose on larval weight gain



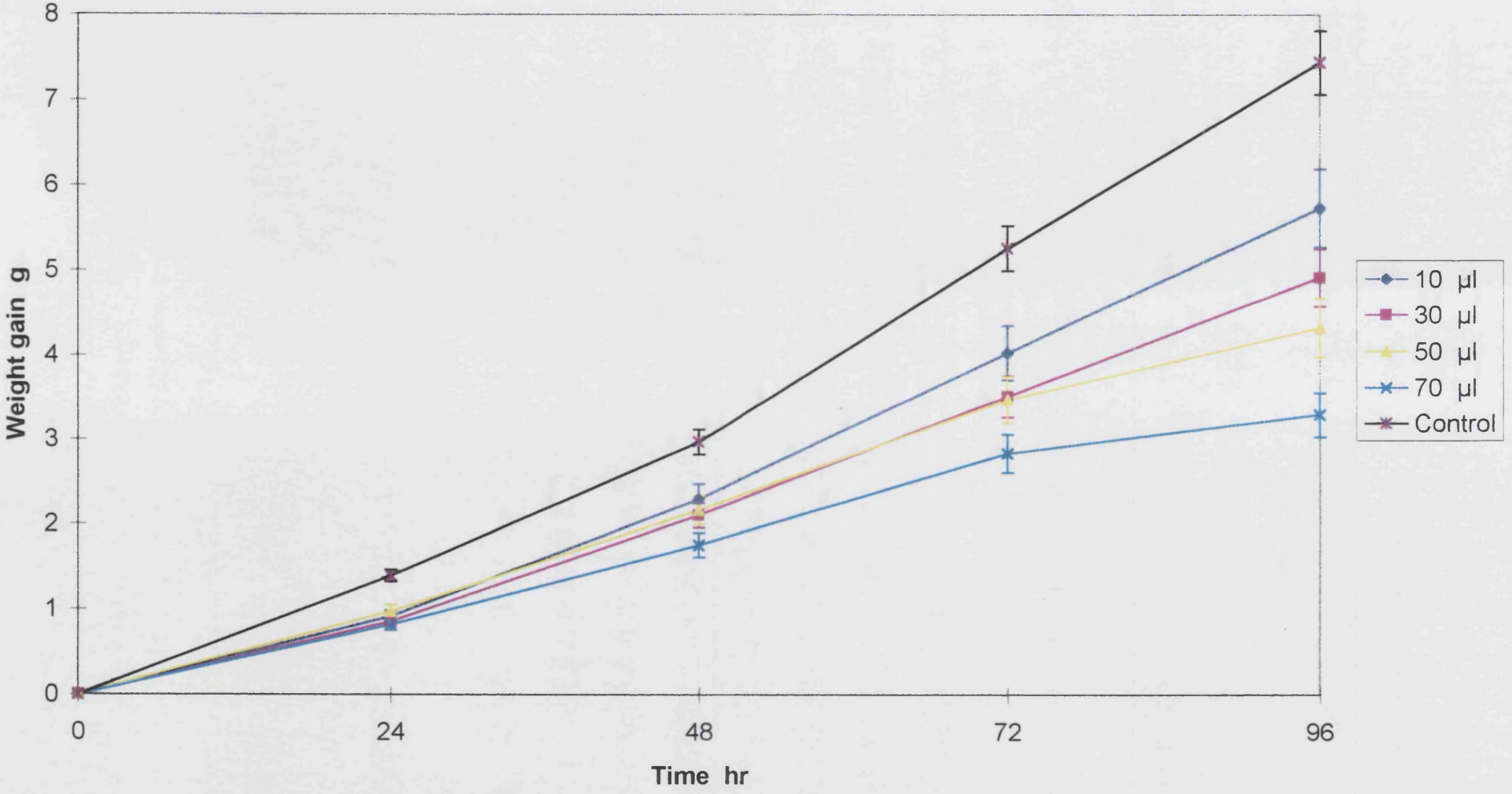
(n = 9 per dose)

FIGURE 14. Effect of 1886 filtrate dose on weight gain



(n = 9 per dose)

FIGURE 15. Effect of 2727 filtrate on larval weight gain



(n = 9 per dose)

3 . 3 . 5 Production of oxalic acid by different isolates of *B. bassiana* grown in liquid medium

B. bassiana isolates produced large amounts of oxalic acid when grown in Czapek-Dox liquid medium. The un-inoculated medium gave a zero reading. Concentration of oxalic acid in the different isolate filtrates varied by a factor of ten (see Table 14). The variation between the different isolates did not appear to be related to their pathogenicity.

The original intention had been to test whether oxalic acid was present or not, so the ability to quantify the amount in different filtrates was a bonus.

Testing the culture filtrates with catalase revealed that there was no free hydrogen peroxide present which might have interfered with the enzyme assay and given a false result.

Table 14.

Concentration of oxalic acid in the culture filtrates of different *B. bassiana* isolates.

Isolate	Amount of oxalic acid mg/l	Ranking
304	>500	1
959	450	6
1007	65	11
1122	>500	1
1315	450	6
1484	480	4
1558	48	12
1629	460	5
1885	488	3
2417	450	6
2727	430	10
3527	445	9

50µl of culture filtrate from each isolate was tested for the presence of oxalic acid by adding 1ml of Reagent A (DMAB and MBTH) and 0.1ml of oxalic oxidase and horseradish peroxidase. The absorbance was read at 590nm and compared with a calibration curve produced with standard concentrations of commercial oxalic acid and the concentration in the different filtrates calculated. The work was repeated 3 times.

3.3.6 Larval weight gain after oxalic acid injection

Some entomopathogenic fungi are known to produce oxalic acid (Wigglesworth 1934) so the effects on larval weight gain of different concentrations of commercial oxalic acid were tested and later compared with the culture filtrates from the growth of *Beauveria bassiana in vitro.*

Five fifth instar larvae were injected with 50µl of oxalic acid solutions. Six different dilutions of oxalic acid were made from 100 to 1000µg per litre. Sterile liquid Czapek-Dox media was used as the diluent, since although it has a high salt level, the weight gain of the larvae could be compared with those treated with fungal filtrate from cultures grown in C-Dox.

Larvae were weighed at 24 hour intervals during incubation at 24°C ± 1°C and their weight gain compared.

Fig.16 (overleaf) shows that larval weight gain is reduced at 72 hours after injection of 50µl of 400mg per litre, or more, of oxalic acid. Analysis of variance showed that this difference was significant ($p<0.05$) suggesting that these concentrations of oxalic acid may disturb the larval growth rate. However, filtrate from ten of the isolates also contained more than 400mg/litre of oxalic acid (see Table 14), yet the injection of fifty microlitres of culture filtrate 2727 (430mg oxalic acid per litre) produced a significantly greater ($p< 0.05$) loss of wieght gain than the injection of 400mg/litre of oxalic acid solution alone. It appears probable that factors in addition to oxalic acid are responsible for the lack of larval weight gain.

Larvae which had gained more than 6g by 96 hr post-injection, went on to pupate normally.

Figure 16

Figure 16 shows the effect on weight gain of *M.sexta* larvae after the injection of different concentrations of commercial oxalic acid.

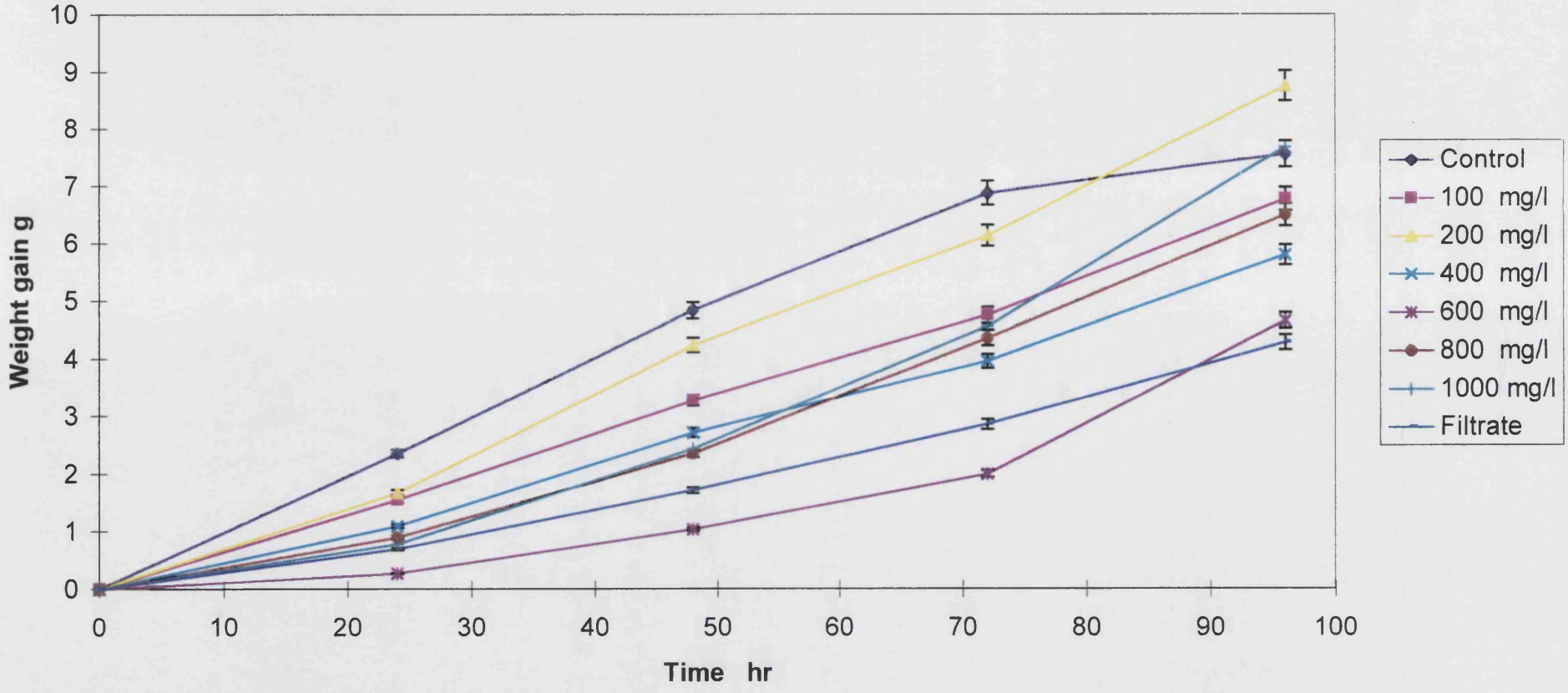
Six different dilutions of oxalic acid, from 100 to 1000mg per litre were made in sterile liquid Czapek-Dox.

Five first day fifth instar larvae were injected with 50µl of the chosen dose, with a further five injected with 50µl filtrate from the culture of isolate 2727 and five injected with sterile Czapek-Dox liquid (controls).

The insects were incubated at 25° C ± 1° C with a 17/7 hour light/dark schedule. They were weighed at 24 hour intervals up to 96 hours.

The work was repeated 3 times (n = 15).

Figure 16. Weight gain of M. sexta larvae after injection with 50 µl of oxalate solutions



(n = 15)

3.3.7 Oxalic acid content of larval haemolymph

The amount of oxalic acid in the haemolymph of injected insects is tabulated below:-

Table 15.

Oxalic acid content of haemolymph after larval injection with fungal spores or oxalate

Haemolymph from:- ↓	24 hr	48 hr	72 hr	96 hr
Infected larva Isolate 2727	0	108 mg/l SEM 9.5	93mg/l SEM 5.7	0
Non-infected larva	0	36 mg/l SEM 2.2	0	0
Oxalic-injected larva 1 mg per insect	5 mg/l	0	0	0

Five fifth instar larvae were injected with 50µl of conidiospores of isolate 2727 at 9.2×10^{6} spores per ml. The amount of oxalic acid in the haemolymph after incubation at 24°C ± 1°C was compared with that of larvae injected with 50µl of a solution of commercial oxalic acid in GIM (1mg per insect).

The concentration of oxalic acid in the haemolymph was determined using proprietary reagents containing DMAB, MBTH, oxalate oxidase and horseradish peroxidase. The results were read at 590nm and compared with a calibration curve.

The work was repeated 3 times.

Larvae infected with *B. bassiana* isolate 2727 gave the highest oxalate reading 48 hours after injection. The reading had dropped by 72 hours and 96 hours after injection no oxalate was found in the haemolymph. However, non-infected larvae also showed a small amount of oxalate in the haemolymph at 48 hours, so this may be produced by normal metabolism. Even quite large injections of artificial oxalic acid did not appear in the haemolymph after 24 hours.

3.3.8 The effects of toxic pigments produced by *B. bassiana* on larval growth

Some isolates of *B. bassiana* are known to produce pigments which have toxic effects on certain insect species. Seven of the isolates tested produced a deep red pigmentation which passed into the media when the fungus was grown on SDA.

When grown in liquid SDA and liquid Czapek-Dox these isolates produced some darkening of the media, but it was difficult to visually separate this from media colour.

PLATE 6

Isolate 1629 showing red pigmentation produced during 8 day growth on SDAY.

The effects on larvae of the seven different *B. bassiana* isolates which produced red pigmentation when grown on plates of SDA were compared with those of an isolate (304) which did not produce this pigmentation. Twenty, first day fifth instar larvae were either injected (50µl), or immersed (10 secs) in suspensions of conidiospores at 1×10^{6} spores per ml. The larvae were then incubated at 25°C ± 1°C for 7 days and their mortality compared with the presence of pigmentation. The work was repeated 3 times (n = 60).

Table 16.

Percentage of 5th instar *M.sexta* larvae which developed pink coloration after treatment with 1×10^{6} spore per ml of isolates of *B. bassiana*

Isolate No.	Injection of 10 µl	Immersion for 10 s
1122	48% (29)	0% (0)
1315	13% (8)	0% (0)
1484	43% (26)	16% (10)
1629	58% (35)	1.6% (1)
2417	23% (14)	0% (0)
2727	73% (44)	16% (10)
3527	41% (25)	0%(0)
304 (Control)	0% (0)	0%(0)

n = 60

Brackets denote the actual number of insects developing pink coloration.

Insects injected with spores from 7 isolates became pink between 72 and 96 hours after injection. Out of 420 insects injected with these isolates, 181 became pink (43%) prior to death, however only 21 out of the 420 insects (5%) immersed in pigment-producing isolates became pink (Table 16). A comparison of insect mortality, with the development of pink colouration, is given in Table 17 overleaf.

The pink colouration occurred throughout the cuticle, though not noticeably on the black or white markings. In insects which continued to grow the colour deepened to carmine, but in those which became moribund and had died by 7 days, the white mycelia outgrew the cuticle and masked the pink coloration. Although many of the insects which became pink had died by 7 days, others continued to grow and pupate normally. Equally, insects injected with isolates which did not produce a pink pigment either on media, or

within the insect, failed to gain weight and died. Neither larval weight gain nor mortality appeared to be linked to isolate pigment production.

Ranking of isolates causing mortality (see Ch. 2.3.1) is compared with those producing pink pigmentation (Table 17).

Table 17.

Comparison of red pigment production and mortality caused by the injection of *B. bassiana* isolates

Isolate No.	* Ranked %Mortality at 7 days	Ranked %Mortality at 7 days	% Presence of Pigment at 7 days
	Injected	Immersed	
304	1	1	0
959	10	9	0
1007	7	6	0
1122	8	9	48
1315	8	9	13
1484	1	4	43
1558	12	7	0
1629	1	9	58
1886	1	4	0
2417	10	7	23
2727	1	2	73
3527	1	3	41

* Percentage mortality ranking from Tables 1 and 2.

Five first day fifth instar larvae were injected or immersed in conidial suspensions of 1×10^{6} spores per ml of each isolate. The larvae were then incubated at 25°C for 7 days. The work was replicated 3 times (n = 15).

There appeared to be no clear relationship between pigment production and mortality ranking of injected spores, though this does not preclude the possibility of synergistic effects of the pigment with other toxic metabolites.

When culture filtrate from the growth of different isolates was injected into *M. sexta* larvae, even though seven of the filtrates had a pink pigmentation, this was not transferred to the larvae even after 96 hours.

It is possible that there was an insufficient concentration of pigment in the culture filtrate to affect the larvae, or that the coloration of the larvae depended on the fungus growing and secreting the pigment within the insect.

3.4 Discussion

3.4.1 Effect of heat-killed spores on larval weight gain

The inoculation of larvae with heat-treated spores showed that heating destroyed any effect of the spores on larval weight gain. It suggests that substances attached to the spore coat, like laminarin, are unlikely to be the cause of lack of larval weight gain and that in the case of *M. sexta* larvae other factors are likely to be responsible.

Genthner *et al.* (1994) observed high mortality of *Mysidopsis bahia* when they were treated by exposure to heat-killed *B. bassiana* conidiospores. However, the crustacean mortality was attributed to high particulate density and not to fungal toxins. Mysids appear to be affected by suspended sediments (Nimmo, Hamaker and Young 1982) and the high mortality rate after treatment with heat-killed spores is more likely to be due to these sediments than to laminarins, which might still have an effect after heating.

It is conceivable that the early effects of spores on larvae are due to substances carried on the spore-coat which are deleterious to larval growth and weight gain. Even if these substances are not strongly toxic to the insects they could account for part of the negative relationship between larval weight gain and spore concentration. The early effect of malaise and lack of weight gain, probably before blastospores or mycelia have formed, becomes greater with increasing spore concentrations.

Laminarins, which are ubiquitous polysaccharides normally extracted from the brown sea-weed *Laminaria digitata*, are able to block the beta-glucan activated pathway of *Limulus amebocyte* lysate (Zhang, Back, Buchardt and Koch 1994). Laminarin sulphate has been shown to have similar anti-coagulant activity to heparin and may be involved in the regulation of cell growth (Miao, Ishaimichaeli, Paretz and Vlodavsky 1995).

Although it is possible that laminarin-like substances exist on the spore surface and also possible that the *M sexta* larvae are able to digest them, it seems more likely that the early effects on larval weight gain are due to fungal metabolites. Even as the spores germinate and start to grow through the cuticle, fungal enzymes could have an effect on larval weight gain and this would of course be dose-related.

3.4.2 Effect of filtrates from fungal growth in different media on insect weight gain

By growing the cultures in different media it was possible to ascertain if there was an effect of media on toxic metabolite production. The different balance of media salts, or the nitrogen to carbon ratio, might encourage production of one metabolite rather than another. However the difference in weight gain between insects treated with culture filtrate from the same isolate grown in two different media was not significant ($p>0.05$).

Novikova, Mitina and Pavlushin (1994) showed that the proportion of carbon to nitrogen, as well as the sources of these elements, could affect the toxin production of *Verticillium lecanii.* The production of mycelia and hence toxic metabolites appeared to improve on media with unrefined components and possibly trace elements were important. However, problems of media variation where organic components are included could affect reproducibility of results.

Where *B. bassiana* was grown in Czapek-Dox and Sabouraud Dextrose liquid media a difference in metabolite production between the two media may have occurred. *Beauveria bassiana* produces a range of known metabolites and probably many more which have not been characterised, but, if there is a difference in fungal metabolite production due to media, the metabolites do not affect *M. sexta* larval growth.

3.4.3 Effects of isolate and filtrate dose on larval weight gain

From the results of the injection of different doses of culture filtrate it was apparent that toxic activity varied between isolates. Since the culture filtrates were sterile and all had been taken from 8-day cultures there was no effect of the living fungi on the larvae.

The variation between the effects of filtrate from different isolates at the same dose level may be due to different quantities of toxic metabolites produced, or to different types of toxins. There may also be a synergistic effect of two or more metabolites on insect weight gain. A significant ($p<0.05$) reduction in weight gain occurred at 24 hours after injection with ten microlitres of culture filtrate from isolate 1315. This did not occur with isolate 2727 at the same dose, but an increased dose of 70 microlitres gave a significant difference ($p<0.02$) at 48 hours. This could be due to differences in metabolite concentration, or to the early production by one isolate of toxins.

Mollier *et al.* (1994) extracted a range of products from a liquid culture filtrate of *B. sulfurescens.* After extraction, one of the fractions induced tetanic paralysis in *G.*

mellonella larvae. The toxicity was reduced on treatment with pronase, suggesting that the toxin was a protein rather than a protease. However he concluded that the crude filtrate contained two or more metabolites with independent toxic activities.

Variation between *B. bassiana* isolates is well established. Champlin and Grula (1979) failed to extract beauvericin from the liquid growth media of *B. bassiana*, but showed that synthetic beauvericin was not toxic to *Heliothois zea*. Frappier, Ferron and Pais (1975) showed that not all *B. bassiana* isolates produced beauvericin, thus strengthening the idea of variation in toxin production between isolates.

Apart from beauvericin, Roberts (1980) lists bassianolide, isarolides, beauverolides, the pigments tenellin, bassianin and oosporein and also oxalic acid as frequently occurring metabolites of *B. bassiana*. Undoubtedly, there are many more metabolites, which, if they do not cause a specific effect, or arise in large concentrations, have passed unnoticed. However, within a filtrate, even in small amounts, these metabolites could have a synergistic effect on insect development. Even a change in pH from the production of oxalic acid could alter the effect of a metabolite so that it became toxic to a particular insect species, or insect stage. Other metabolites may remain bound to the mycelial wall, or become quickly broken down during culture growth. They may even be produced later when the culture reaches senescence and so would not normally be extracted.

Metabolite production in artificial media is inevitably different from metabolite production *in vivo*. Even so, the filtrate products can give an insight into the mechanisms with which *B. bassiana* attacks its host.

Since the results were clearly dose-related for some of the isolates, it may well be that these isolates are able to affect *M. sexta* not only by mycosis, but by the toxins which reduce the rate of weight gain and delay metamorphosis.

3.4.4 Effect of mycelial production on culture filtrate toxicity

Since the filtrates were obtained from cultures grown in liquid media where metabolites had passed from the mycelia into the culture medium, it was conceivable that isolates with the best mycelial growth would produce the greatest amount of metabolites. However,there was no significant difference ($p>0.05$) between the dry weights of mycelia from the growth of the twelve different isolates.

Although this may be so there was no obvious relationship between isolates producing large amounts of mycelia and the effect of the filtrate on the *Manduca sexta* larvae. It may be that isolates producing large amounts of mycelia only produced small amounts of toxic metabolites, or that slower growing isolates produced their toxic metabolites later.

One might expect that culture filtrates from the isolates with the greatest mycelial dry weight would produce the greatest effect on the larvae, but there was no evidence to support this.

It is possible that isolates able to grow vigorously do not produce large quantities of toxic metabolites and that their success as pathogens depends on mycosis. Hence the good producers of mycelia may not be the good producers of toxins, and the isolates producing the greatest toxic effect on larvae may only form moderate amounts of mycelia.

3.4.5 Effect of oxalic acid on larval weight gain

When the weight gain of larvae after immersion or injection, of spores (earlier work) was compared with the amount of oxalate produced in the filtrate, the two isolates producing the most oxalic acid (304 and 1122) also produced the lowest weight gain after immersion in spore suspensions. Oxalic acid may be able to solubilise the insect cuticle and aid fungal entry. However, it seems unlikely that there would be sufficient on the surface of the spores to have this effect since oxalic acid is mainly produced by mycelia. The fact that the two isolates which produced the largest amount of oxalic acid are also pathogenic by immersion to larvae may be coincidental.

There was a significant ($p<0.05$) relationship between oxalic acid concentration and percentage mortality with two isolates. Isolate 304 had the highest percentage mortality after injection (Table 2, page 30) and the highest oxalic concentration (Table 14, page 96) whilst isolate 1558 caused the lowest mortality and had the least oxalic acid. There was no significant relationship ($p>0.05$) between oxalic acid production and pathogenicity with any of the other isolates.

Although it seems most probable that the oxalate in the haemolymph of infected insects was of fungal origin, Wigglesworth (1934) states that crystals of calcium oxalate can occur in the Malphigian tubules of lepidopteran larvae. This oxalate may originate from preformed oxalic acid in the insect's food, but the presence of crystals

suggests that the larvae were able to tolerate high concentrations, hence a tolerance of high oxalate levels of fungal origin is not surprising.

One might expect the most obvious effect of oxalic acid to be a reduced weight gain from larvae immersed in spore suspensions of isolates producing high amounts of oxalic acid, since this might solubilise the cuticle and allow early fungal penetration and subsequent rapid growth within the insect. However, the results suggested that although this might occur, other factors were probably more important, since low concentrations of oxalic acid did not result in rapid larval growth.

It would appear that if oxalic acid plays any part in causing malaise or lack of weight gain in *Manduca sexta* larvae it must be in conjunction with other fungal products and that the insect is able to tolerate, or neutralise, considerable quantities of oxalate with little obvious ill-effect.

3.4.6 Effects of toxic pigments on larval growth

B. bassiana produces several characterised pigments, of which oosporein is the best known. This dibenzoquinone is produced by several fungi including many isolates of *B. bassiana*, but the production of the red pigment can be lost after repeated transfer of isolates on artificial media (Roberts 1980).

B. bassiana also produces the yellow pigments, tenellin and bassianin, which are concentrated in the mycelium, whereas oosporein is released into the media. This allows it to be readily taken up by the organisms treated with the fungus and its red colour can be easily traced. Cole, Kirksey, Cutler and Davis (1974) showed that oosporein was toxic to day-old cockerels, but Roberts (1980) suggested that its toxicity to insects was unknown.

Eyal *et al.* (1994) thought that oosporein might be a useful mycotoxin for whitefly and mealy bugs. He proposed that it might act as an antagonist to intestinal flora and allow fungal mycelia to develop more rapidly. The strains of *B. bassiana* that he used were highly virulent against whitefly and mealy bug however he did not correlate the percentage mortality of the insects with the pigmentation due to oosporein. He concluded that the red pigmentation of the insects could be used as a fungal infectivity indicator and that the anti-bacterial activity of oosporein might assist fungal growth, but he did not confirm the toxicity of oosporein to whitefly or to mealy bug.

The pink pigment produced by seven of the *B. bassiana* isolates tested against *M. sexta* larvae was released into the insect cuticle. However the pigmentation appeared

to be limited to the cuticle and was not noticeable in the haemolymph of insects infected with these strains. It is possible that the red colouration was masked by the blue-green colour of the haemolymph, but since there was no difference in the colour of the experimental or control insect haemolymph it is likely that the red pigmentation was not present.

In the use of *B. bassiana* against *M. sexta* larvae, percentage mortality (Table 17, page 103) did not correlate with the red pigmentation of the insects. Additionally some of the most pathogenic strains did not produce oosporein at all so it was of little use as a fungal infectivity agent. Even if it had allowed infection to develop more quickly than in oosporein-deficient isolates, one would have expected the larvae to have died of mycosis at an early stage, which was not the case.

The colour coding of pathogenic isolates with their toxic pigments would be an attractive way of following their effects, but without genetic engineering, oosporein is not the pigment to provide this tool. Even if used as a discriminator between applied and naturally occurring isolates oosporein would be unreliable since natural isolates might mutate and develop the pigment, or applied isolates might lose the ability to produce it.

Chapter 4

The effect of *Beauveria bassiana* conidiospores and fungal filtrate on *Manduca sexta* haemolymph

4.1.1 Introduction

Both the conidiospores of isolates of *Beauveria bassiana* and the filtrate from *in vitro* growth of the fungus have been shown to affect the larval weight gain of *Manduca sexta* (2.3.3. and 3.3.2c). A study of their effects on the larval haemolymph might reveal their mode of action and help to determine why some isolates are more pathogenic than others.

Insect haemolymph has two distinct ways of combating infection and invasion, humoral and cellular. Humoral responses include the production of anti-microbial peptides and proteins and are reviewed in Chapter 1. Since so much is known about the humoral responses of insects,the current work concentrates on the cellular responses. In Chapter 4 attention is focussed on the responses which involve phagocytosis, nodule formation and encapsulation.

4.1.2 Cellular responses

Insect haemocytes respond to invasion in three recognised ways. The invading material may be engulfed and phagocytosed; it may stimulate the formation of haemocytic nodules, or it may result in the formation of many-layered melanised capsules.

Many insect species demonstrate these responses to a wide range of invading materials including bacteria, fungal spores, eggs of parasitic wasps and inanimate materials like silica beads (Lackie 1983, Vilcinskas, Matha and Götz 1997).

Although phagocytosis is a common mechanism for the removal of foreign bodies from both vertebrate and invertebrate haemolymph it is not always followed by the successful breakdown of the ingested material. Vilcinskas *et al.* (1997) showed that *Galleria mellonella* plasmatocytes were able to ingest *Metarhizium anisopliae* hyphal bodies, but were not able to break them down and that the hyphal bodies were able to divide and outgrow the engulfing haemocytes.

Insect haemocytes may simultaneously phagocytose invading material and also form multi-cellular nodules (Ratcliffe and Walters 1983). The rate and type of reactions

appear to depend on the pathogenicity and dose of the organism involved. The more pathogenic organisms cause the early formation of large nodules at lower doses than the less pathogenic organisms. Ratcliffe and Walters (1983) were able to show a clear dose dependence for nodule production by *Galleria mellonella* larvae in response to *Bacillus cereus.*

Gunnarsson and Lackie (1985) showed that both the locust, *Schistocerca gregaria* and the cockroach, *Periplaneta americana* produced haemocytic nodules after injection with *M. anisopliae* conidiospores. In addition they showed that Zymosan (cell walls from the yeast *Saccharomyces cerevisiae)* and Laminarin (algal oligomer) induced nodule formation.They suggested that:-

1) co-agulocytes have cell membrane receptors which induce de-granulation when activated

2) supernatant from spore suspensions was as effective after heating as before in stimulating nodule formation.

Ratcliffe and Gagen (1976) proposed that nodule formation by haemocytes followed a recognisable sequence. They suggested that events on the cell surface triggered cellular defence reactions. Firstly, the granular cells discharged some of the contents of their vacuoles including agglutinins and lysozyme. The discharged material appeared to be sticky and to cause the early aggregation of granular cells (GRs). After de-granulation the GRs disintegrated and attracted plasmatocytes (PLs) which formed an envelope around the GRs. Eventually a large multicellular envelope of flattened haemocytes developed with a centre composed predominantly of GRs. Some hours after the commencement of nodule formation melanisation occurred. Typically this started in the centre of the nodule and expanded to the outer layer of cells until the complete capsule was melanised. The size of the capsule was eventually limited by the thickness of the cell cover, since once the GRs were covered by layers of PLs the signal from the active GRs was attenuated.

The formation of nodules may well be stimulated by the surface of the invading material. Hung, Boucias and Vey (1993) found that *Beauveria bassiana* blastospores produced *in vitro* and injected into *Spodoptera exigua* larvae did not stimulate the production of haemocytic nodules, but were rapidly phagocytosed by granular cells. Yet *Candida albicans* yeast cells became localised within multi-layered haemocytic nodules six hours after injection. They suggested that this difference might

be due to the lack of elicitor production by the *B. bassiana* blastospores, or even the production of immunosuppressive substances since the fungus is known to be able to suppress haemocyte-pseudopodial spreading.

Specific factors may be involved in nodule formation. Huxham, Lackie and McCorkindale (1989) showed that glucan-induced nodules formed by haemocytes of *Periplaneta americana* and *Schistocerca gregaria* could be suppressed by mixtures of destruxins. These cyclic depsipeptides are produced by the fungus *Metarhizium anisopliae*. It is quite possible that *Beauveria bassiana* produces similar metabolites which suppress haemocyte nodulation and act on insect blood cells in a comparable way.

In 1996 Lavine and Beckage looked at the way in which the encapsulation of the parasite eggs of *Cotesia congregata* by *Manduca sexta* larval haemocytes was suppressed. It appeared that the polydnavirus carried by *C. congregata* could prevent the host haemocytes from forming nodules and allow the parasitoid eggs to avoid encapsulation.

Lavine and Beckage (1996) were also able to show that the polydnavirus was able to prevent encapsulation of Sephadex A-25 beads, but they did not go on to isolate the encapsulation-suppression factor. Whilst the effect of the factor was clear-cut the isolation of a single metabolite from the complex interaction proved difficult.

Mandato, Diehl-Jones, Moore and Downer (1997) looked at specific inhibitors of nodule formation in the haemolymph of *Galleria mellonella* injected with latex beads. They showed that dexamethasone, indomethacin and esculetin were all able to inhibit nodule formation, but that arachidonic acid was able to rescue the effect of dexamethasone and that on its own it promoted nodule formation. Interestingly esculetin and indomethacin both also inhibited phagocytosis, suggesting that the two cellular responses of nodule formation and phagocytosis might in fact be linked.

Stanley-Samuelson, Pedibhotla, Rana, Rahim, Hoback and Miller (1997) and Miller, Howard, Rana, Tunaz and Stanley (1999) were able to show that in two different species of insects, *Bombyx mori* and *Gryllus assimilis*, injected with the bacterium *Serratia marcescens*, nodulation could be reduced by naproxin and dexamethasone. Arachidonic acid could reverse the effect and promote nodulation.

The cellular responses of insect haemolymph to fungal infection have been less-well studied than responses to bacteria. Yet the use of fungal cells could help to reveal the method of interaction between insects and entomopathogens and to clarify

why some micro-organisms are pathogenic to insects whilst others are not. Insects of different species produce a large range of metabolites in response to infection yet cellular reactions are remarkably similar, hence a study of the cellular responses of one species of insect may provide a model for others.

4.1.3 Aims and Objectives

The aims of this part of the study centred on the effects of *Beauveria bassiana* spores and filtrate on the haemolymph of *Manduca sexta* larvae.

The first objective was the identification of the major haemocyte classes using Wright's Stain. These results could then be used to verify the identification of immuno-stained haemocytes. The use of antibodies to haemocytes of *Galleria mellonella* as well as *M. sexta* would show if cross-specific staining occurred.

The next objective was to determine whether the reaction of the larval haemocytes was linked to the pathogenicity of different *B. bassiana* isolates.

Fungal filtrate from *in vitro* growth of *B.bassiana* had been shown to reduce larval growth rate. A study of the effects of the fungal filtrate on phagocytosis and nodulation would show if the filtrate could also alter cellular reactions.

The eicosanoids and prostaglandins are known to affect cellular reactions. A comparison of dexamethasone, indomethacin and arachidonic acid with the fungal filtrate from the growth of *B. bassiana in vitro* might indicate whether it had a similar effect on the haemocyte monolayers.

Studies on the characteristics of the filtrate components which are able to alter cell reactions might lead to a better understanding of the mode of action of the fungus *in vivo*. To this end size, heat stability and reaction to protease would provide useful data.

If protease alters the effects of some filtrates on larval haemocytes a comparative study of the protein components of the different filtrates might indicate which isolates produced immuno-suppressive proteins.

4.2 Materials and Methods

4.2.1 Insects

Only 1d 5L *Manduca sexta* larvae, weighing between 1.0 and 1.6 g, were used in this section. The same stage larvae were consistently used to prevent anomalous effects from the changing levels of metabolites towards the end of the larval stage.

Injections for *in vivo* work were made laterally and slightly to one side of the mid-line into the inter-segmental muscle between the seventh and eighth segment of the larvae. A 25 gauge 100 µl Hamilton syringe was used and the needle rinsed in 70% ethanol and sterile distilled water between injections.

After treatment, insects were fed *Manduca* diet (Appendix) and incubated at 25° C ± 1° C, 100% humidity and 17/7 light/dark schedule (see Section 2.2.3).

4.2.2 Fungal Culture

Spores of *Beauveria bassiana* were prepared as in Section 2.2.1. They were suspended in 10% glycerol and stored at -20° C. Prior to use they were thawed, centrifuged at 1400*g* for 15 minutes and then the pellet was washed 3 times with sterile distilled water. The spores were resuspended in Grace's Insect Medium (GIM) and counted on a Neubauer Improved haemocytometer.

Fungal filtrates were prepared as for Section 3.2.3, divided into small aliquots and stored at -20°C. Before use, the filtrates were thawed and passed through Acrodiscs (Gelman Sciences) with 0.8/0.2 µm pore size, to ensure sterility.

Once thawed the remains of used aliquots were discarded and not re-used.

4.2.3 Haemolymph and Monolayers

A series of experiments was carried out to establish a reliable protocol for removing haemolymph from larvae, establishing monolayers, recognising different cell types and counting nodules. Since insect haemocytes are fragile every effort was made to prevent damage to them which might alter the effect of the treatments.

The osmolality of larval blood was established using an Automatic Roebling Osmometer (Camlab). Six 1d 5L larvae were individually bled and the osmolality of the haemolymph measured without anticoagulant to give a mean of 323.6 osmols with a

standard deviation of 1.14. Subsequently all final dilutions of spores or other substances to be added to monolayers, were made in GIM with an osmolality of 299 mOs per kg.

Prior to bleeding, the larvae were held at 4° C for 5 minutes. They were then wiped with 70% ethanol, and the horn removed with flamed scissors.

Different methods of haemolymph collection were tried. These included injecting larvae with anticoagulant prior to bleeding, and using different quantities of anti-coagulant in the collection tubes.

However, the most consistently successful method and that least likely to interfere with subsequent reactions, was to allow the haemolymph to drip directly into 1.5 ml microcentrifuge tubes containing 30 µl of anti-coagulant buffer (see Appendix) at 4°C.

The volume of haemolymph from 6 first day fifth instar larvae was approx. 100µl per insect. At 5 days (5d5L) the volume had risen to 630µl per insect so to avoid volume discrepancies first day fifth instar larvae were always used.

The microcentrifuge tubes containing haemolymph and anti-coagulant were gently inverted and centrifuged at 77 *g* for 5 minutes, the supernatant removed and replaced with an equivalent amount of GIM at 4° C. Centrifuge speeds were set as low as possible (77*g*) to prevent cell damage, but still obtain a pellet and all additions to monolayers were held at 4° C until used.

After centrifugation the resuspended cells were placed in paired wells on glass cavity slides for further treatment.

All treated slides were incubated at 22° C ± 1° C in an humidity chamber at 100% R.H.

4.2.4 Recognition of haemocyte types and enumeration

Both conidiospores and haemocytes were counted using an Olympus BH2 microscope with × 40 objective lens and phase contrast illumination. Monoclonal antibodies to *M. sexta* haemocytes for staining and distinguishing cell types were the kind gift of Dr M. Kanost of the Department of Biochemistry, Kansas State University, U.S., whilst antibodies to *Galleria mellonella* haemocytes were kindly given by Professor Norman Ratcliffe of Swansea University.

Dr Kanost's antibodies were produced by immunising female Balb/c mice with washed haemocytes from 5th instar *Manduca sexta* larvae. The mice were injected

again at 3 and 7 weeks after the first injection. Three and a half days after the last injection the spleens were harvested.

The spleen cells were fused with X63Ag8.653 myeloma cells and incubated in RPMI medium. Hybridoma cells producing antibodies were detected using enzyme-linked immunosorbent assays (ELISA). Hybridoma supernatants were dialyzed with 100 kDa cutoff and were used to stain *M. sexta* haemocytesas described in 4.2.6.

Professor Ratcliffe's antibodies were prepared by immunising Balb/c female mice with purified haemocyte types of *Galleria mellonella.* The insect haemocytes had previously been separated on 60% Percoll gradients.

Mice were immunised on Day 0 and Day 14 with insect haemocytes. They were tail bled on Day 28 and the antibody titre tested by ELISA. Mice with a satisfactory titre were given a booster injection after 4 weeks and the spleens harvested three days later.

Spleen cells were fused with NSI myelomas and the subsequent hybridomas grown in RPMI 1640 medium. Hybridomas secreting the appropriate antibodies were selected by ELISA. Culture medium from the growth of the anti-body producing hybridomas was used to stain haemocyte monolayers as in 4.2.6.

Complete details of antibody preparation are given in papers by Mullet, Ratcliffe and Rowley (1993) and Willot, Trenczek, Thrower and Kanost (1994).

For recognition of cell types using Wright's stain and antibody staining, the number of fixed cells in ten fields at ×40 magnification were counted. Total haemocyte count (THC), plasmatocytes (PL's), granular cells (GR's) and oenocytoids (O's) were scored. For phagocytosis and nodule-formation work, live cells were counted using 10 fields of view, unless otherwise stated. Where spores were stained with rhodamine, phagocytosis was observed both under phase contrast and under U.V. light using a fluorescent attachment and a blue-green excitation filter, with an Olympus BH2 RFL microscope.

Generally, the observation of live cells on cavity slides under phase contrast gave the best conditions to interpret cell reactions. This also allowed the same cells to be viewed as a reaction progressed and changes occurred which would not have been possible with fixed cells.

4.2.5 Glassware and reagents

To avoid endotoxin contamination all glassware in contact with insect cells was prepared in accordance with Sigma Technical Bulletin 210.

The glassware was soaked overnight in a 1% solution of E-Toxa-Clean (Sigma), rinsed 8 times in running water and 8 times in pyrogen-free water. It was then dried in a hot air oven, wrapped in aluminium foil and heated to 180° C for 4 hours.

All media, reagents, buffers and stains, unless otherwise stated, were obtained from Sigma-Aldrich Co. Ltd. Poole, Dorset, U.K. and were made up in sterile endotoxin-free distilled water.

Final dilutions for larval injection and addition to haemocytes *in vitro* were made in GIM to preserve osmotic balance.

4.2.6 Antibody staining of larval haemocytes

Monoclonal antibodies from two different sources (see 4.2.4) were used to differentially stain haemocytes. Gardiner and Strand (1999) showed that some antibodies to haemocytes of the soybean looper, *Pseudoplusia includens*, stained all classes of haemocytes, whilst others only stained sub-populations of plasmatocytes, so morphological examination and the use of Wright's stain were employed to identify cell types and as a comparison with antibody staining.

Haemolymph was collected from 7 × 1 day fifth instar larvae and monolayers prepared on cavity slides as in 4.2.3. The monolayers were incubated for 30 minutes at 22°C ± 1°C. Cells were allowed to settle and control slides counted to check for loss of any classes of haemocytes. Cells were fixed for thirty minutes with 4% formaldehyde in GIM and then washed three times with GIM. After this 3% hydrogen peroxide in 10% methanol was added to the monolayers to destroy any endogenous peroxidase enzyme which might be present in the cells and which could give a false positive result.

Control slides were stained for 90 seconds with Wright's stain (Sigma) composed of 0.1 g of powdered dye dissolved in 60 ml of 100% methanol and diluted 1:4 in phosphate buffer saline (PBS). Slides were then rinsed with distilled water and total and differential cell counts made.

Antibody staining of haemocytes followed the protocol of Mullett *et al.* (1993) but was modified for *M. sexta* haemolymph on cavity slides.

Monolayers were prepared as before and, after the removal of hydrogen peroxide, were rinsed three times with PBS. They were then blocked with a solution of 3% foetal calf serum; 100mM glycine; 10% goat serum; 0.05% Tween 20 and 1% Triton 100 and incubated for 30 minutes at 22° C.

The blocking agent was removed and a one-to-one solution of antibody in the blocking solution without Triton 100, was added to each well. The monolayers were incubated for 1 hour at 22° C after which the antibody was removed and the monolayer washed three times for 5 minutes each with PBS.

The secondary antibody, a 1 in 500 dilution of goat-anti-mouse antibody, labelled with peroxidase in blocking buffer was added to the monolayers and they were then incubated for 1 hour at 22° C.

After incubation, the secondary antibody was removed and the monolayer washed three times with PBS, as before. The monolayer was then stained in the dark for 15 minutes with a 1:1 solution of diaminobenzidine hydrochloride (DABS, Sigma) in 0.02% hydrogen peroxide with two crystals of cobaltous chloride ($COCl_2.6H_2O$, Fisons, Loughborough) added.

When the stain was sufficiently developed the reaction was stopped by rinsing three times with PBS. The slides were air-dried, examined under phase contrast and the cell types counted. All seven stains were used on separate monolayers and replicated three times.

Staining haemocytes from infected larvae

Mullet *et al.* (1993) suggested that antibody labelling of haemocytes might alter their ability to react to infection and to attach to bacteria. Consequently a study was made to see if infection with *B. bassiana* would alter the labelling characteristics of the antibodies and haemocytes.

Seven larvae were injected or immersed with a spore suspension of isolate 2727. The spores had been prepared as in 4.2.2. Fifty µl of 3.7×10^6 spores per ml in GIM were injected into seven 1d5L larvae which were then incubated for 4 hours at 24° C ± 1° C and 100% humidity. Alternatively larvae were dipped in a spore suspension of 2.3×10^8 spores per ml prior to incubation for 72 hr at 24° ± 1° C.

After incubation the larvae were sacrificed and the haemocytes stained with antibodies as before.

The work was replicated three times.

4.2.7 Germination of spores in haemocyte suspension in GIM

It had already been shown (Table 3) that there existed a variation between the LT_{50} of larvae injected with different isolates, even though the ability to penetrate the cuticle was not in question.

However, the ability of conidiospores of different isolates to germinate and grow in larval haemocyte suspension needed to be investigated before a valid comparison of the mode of pathogenicity between isolates could be made.

Monolayers of haemocytes were prepared as in 4.2.3 for each isolate, with 30 µl of conidiospores at 5×10^6 spores per ml added to each monolayer. These were then incubated in a moisture chamber at 24° C and 100% RH and counted at 8 hour intervals

At least 200 spores were counted on each monolayer and each count of the twelve isolates was replicated three times. The ratio between germinated and non-germinated spores was calculated and the isolates ranked according to germination rates at 24 and 48 hours.

4.2.8 Phagocytosis studies

Ratcliffe and Walters (1983) considered phagocytosis as the primary cellular response of *Galleria mellonella* to low doses of pathogenic organisms. They showed that the phagocytic response was related to the pathogenicity of the microbe, but that it did not increase with dose. Indeed, with doses above 1×10^3 microorganisms per µl, nodule formation confused the results.

Hung *et al.* (1993) found that after infection of *Spodoptera exigua* larvae with *Beauveria bassiana* the phagocytic competence of circulating haemocytes was reduced. They showed that filopodial formation by granular cells was reduced and suggested that fungal metabolites might be responsible.

In order to see if this effect also occurred in *M. sexta,* a series of assays was undertaken to quantify the ability of haemocytes to phagocytose *B. bassiana* conidiospores Originally monolayers were set up as in 4.2.3 using isolates 2727, 959 and 2417, with 20 µl of conidiospores at 4×10^6 spores per ml added to each monolayer.

Although the adhesion of spores to haemocytes was visible under phase contrast it was difficult to determine if the spores had been ingested or were merely adhering to the haemocyte surface. A method using fluorescently-labelled conidiospores was followed (Rohloff, Weisner and Götz, 1994), which could clarify whether spores were ingested or merely adherent.

Stored conidiospores were thawed and centrifuged at 1407*g* for 15 minutes. The pellet was washed twice with 0.01% Tween and then twice with carbonate buffer (see Appendix).

The washed conidia were stained with a solution of 50 mg of rhodamine B isothiocyanate (Sigma) dissolved in 50ml of sterile carbonate buffer. Staining continued for 16 hr at 4° C on an electronic shaker (1KA VIBRAX VXR) with 200 oscillations per minute, in the dark.

The conidia were then washed 5 times with 0.01% Tween in covered tubes to exclude the light. Stained spores were diluted in GIM and counted to give 6×10^5 spores per ml.

Monolayers of haemocytes were prepared from 10 × 1d5L larvae (as in 4.2.3) and 10 µl of rhodamine-stained spores of isolate 2417 suspended in GIM were added to each well. To ascertain the effect of fungal culture filtrate on phagocytosis, 10 µl of culture filtrate from isolate 304 was added to the experimental cells. Czapek Dox liquid medium was added to control wells. The monolayers were then incubated in the dark at 24° C ± 1° C, 100% RH for 4 hr.

After incubation, 40 µl of the culture media was exchanged for 40 µl of trypan blue (Sigma). The dye was made up at 2 mg per ml in GIM with pH 6.5 as recommended by Rohloff *et al* (1994). The fluorescence from non- phagocytosed spores was quenched after 20 min. The monolayers were then rinsed three times with GIM. They were examined under an Olympus BHC microscope fitted with a reflected-light fluorescent attachment, a green-blue exciter filter, a green dichroic mirror and a 610 barrier filter at × 400.

THC, haemocytes with phagocytosed fluorescent spores and nodules were counted over 10 fields.

Each assay had 6 control monolayers and 6 experimental monolayers and was replicated 4 times (n = 24).

4.2.9 Effect on haemocytes of conidia from different isolates

In vivo

A series of experiments was carried out to ascertain whether the conidiospores of the 12 different isolates of *B. bassiana* had varying effects on *M. sexta* larval haemolymph. For each isolate, conidiospores were prepared as in 4.2.2 with 50 µl of spore suspension of 2×10^6 spores per ml in GIM injected into each larva. Insects were incubated at 23° C ± 1° C and 92% RH for up to 6 hours. They were sacrificed at 1,2,4 and 6 hour intervals and the haemolymph was collected and treated as in 4.2.3.

Counts of total haemocytes (THC), plasmatocytes (PL), granular cells (GR), oenocytoids (O) and nodules (N) were carried out on two insects for each isolate at each time point and were replicated 3 times with a total n = 6 for each time point and n = 24 for each isolate

In vitro

The *in vivo* work was followed with a similar series testing the effect of conidiospores of different isolates *in vitro*. Here, 10 µl of 5×10^5 conidiospores per ml in GIM were added to monolayers of haemocytes from individual larvae (haemocytes not pooled). They were incubated for up to 6 hours at 22° C in a moisture chamber at 100% RH. Counts were carried out, as above *in vivo,* with 3 replicates of 3 insects for each time point.

The *in vivo* and *in vitro* results were regressed against the results for LT_{50} of larvae injected with spores of the different isolates (2.3.1 Table 3).

4.2.10 Effect of conidial concentration on nodule formation

Ratcliffe and Walters (1983) showed that the concentration of pathogenic bacteria affected the nodule-forming responses of *Galleria mellonella* larvae. They showed that this depended not only on the pathogenicity of the bacteria, but also on the concentration. With this in mind, trials were carried out to see if the concentration of *Beauveria bassiana* conidiospores affected nodule formation in *Manduca sexta* larval haemolymph.

Spores of two different isolates, 304 and 2417 were prepared as in 4.2.2.

After counting on a Neubauer haemocytometer the spores were diluted in GIM to give four concentrations :-

5.3×10^7

5.3×10^6

5.3×10^5

5.3×10^4

Monolayers of haemocytes from individual caterpillars were prepared as in 4.2.3 and 10 µl of spore suspension was added to each 90 µl of haemolymph. The monolayers were then incubated at 22° C ± 1° C in a humidity chamber at 100% RH.

Six replicate monolayers were used for each concentration. Ten fields were examined for each monolayer using an Olympus BH2 microscope with phase contrast at × 400 magnification. Counts were made of total haemocytes, plasmatocytes, granular cells, oenocytoids and nodules.

4.2.11 Effect on haemocytes *in vivo* and *in vitro* of heat-killed conidiospores

It has been suggested that the insect cellular reaction to infection is principally to the peptidoglycan of microbial cell walls and not to live microorganisms.

Horohov and Dunn (1982) showed that the increase in Total Haemocyte Count (THC) following the injection of *Manduca sexta* larvae with the formalin-killed bacteria *Pseudomonas aeroginosa* was not significantly different from that of viable bacterial cells. This reinforced the suggestion that insect reaction was to cell wall material and not to live microbes.

By comparing the effect of heat-treated and non-heat-treated spores *in vivo* and *in vitro* it is possible to show whether the haemocyte reaction is to fungal wall material or to fungal growth. Additionally the comparison would demonstrate whether the response was from the whole animal, or uniquely from the haemocytes.

Fungal preparation

Conidiospores of *Beauveria bassiana* isolates 304 and 2417 were prepared as in 4.2.2 but were suspended in sterile distilled water. The cultures were each divided into two equal aliquots and one aliquot from each isolate was autoclaved at 120° C for 20 min. After cooling the spore suspensions from heated and non-heated aliquots were streaked onto SDAY plates to check for conidial viability. The plates were incubated at 25° C ± 1° C for 5 days and then examined.

The remaining spores from all 4 aliquots were diluted in GIM, counted and adjusted to give a concentration of 3×10^5 spores per ml.

In vitro

Haemolymph monolayers were prepared from 10 × 1d5L larvae as in 4.2.3. To each 30 µl of haemolymph in a monolayer, 10 µl of conidiospores of isolates 304 or 2417 in GIM was added. GIM alone was used for the control.

Monolayers were incubated for 4 h in an humidity chamber at 22° C ± 1° C and 100% RH. The haemocytes were then counted under phase contrast at × 400 and scored for THC, PLs, G's, O's and nodules.

In vivo

The same heated and non-heated spore suspensions were used as *in vitro.* Two 1d5L larvae were used for each treatment and were injected (as in 4.2.1) with 50µl of 3×10^5 spore suspension in GIM. Control insects were injected with 50 µl of GIM.

All the treated larvae were then incubated for 4 hr at 25° C ± 1° C with 100% RH, and a 17/7 hr light/dark schedule. At the end of incubation the insects were bled, monolayers prepared and counted as in 4.2.3.

Both *in vivo* and *in vitro* work was repeated twice (three replicates), each with paired insects or monolayers to give n = 6 for each treatment.

4.2.12 Effect on haemocyte monolayers of the culture filtrate from different isolates

The fungal filtrate of 12 isolates of *Beauveria bassiana* had already been shown to reduce weight gain in *Manduca sexta* larvae in a dose-dependent manner (3.3.2c). Its effect on insect haemocytes might help to explain why some isolates were more effective pathogens than others.

Culture filtrate from isolates 2417 and 304 were re-filtered through 0.8/0.2 µm Acrodiscs (Gelman Sciences) to ensure that no fungal spores or mycelia remained. They were also streaked on SDAY plates which were incubated at 24° C ± 1° C for 5 days to check for fungal growth.

In vitro

Monolayers from 6 × 1d5L larvae were set up as in 4.2.3 and 20 µl of culture filtrate from isolates 304 or 2417 was added to the experimental wells. Czapek Dox and GIM were used for controls.. The prepared monolayers were incubated for 4 hr at 22°

C ± 1° C and the haemocytes and nodules from 10 microscope fields (at × 400) quantified.

In vivo

To compare filtrate effects on the haemolymph *in vitro* and *in vivo* 6 × 1d5L were injected with 50 µl of culture filtrate from isolates 2417 or 304, and then incubated at 24° C ± 1° C for 4 hr at 100% RH. The larvae were then sacrificed (as in 4.2.3), monolayers prepared and counted. Six insects were used for each essay and this was repeated 3 times (n = 18).

4.2.13 Effect of adding culture filtrate to haemocyte monolayers with isolate 2417 spores

Haemocyte monolayers incubated with *B. bassiana* spores of some isolates produced haemocytic nodules. The addition of fungal filtrate to the monolayer might alter this reaction.

Monolayers were prepared from 1d5L larvae as in 4.2.3. To each 60µl of haemocytes in GIM, 10 µl of isolate 2417 spores at 1.9×10^5 spores per ml in GIM and 10 µl of culture filtrate were added. Culture filtrates from all twelve isolates were tested. Ten microlitres of liquid Czapek-Dox medium replaced the filtrate in one set of control wells, whilst 10 µl of GIM replaced spore suspensions in the other.

The monolayers were incubated in an humidity chamber at 22° C ± 1° C and 100% RH for 4 hr. Total haemocyte count and number of nodules in 10 fields at × 400 was recorded. Twelve larvae were used and the work replicated 3 times (n = 36).

4.2.14 Effect of the concentration of culture filtrate of isolate 304 on nodule formation *in vitro*

Since culture filtrate of isolate 304 appeared to prevent nodule formation by haemocytes incubated with isolate 2417 spores (see Table 28), the effect of reducing the concentration of the filtrate was investigated.

Monolayers of haemocytes in GIM were set up as in 4.2.3 and 10 µl of isolate 2417 spores at 2.4×10^5 spores per ml added to each well.

Culture filtrate of isolate 304 was diluted with sterile GIM as follows:-

whole filtrate; 1/5; 1/10; 1/25; 1/50; 1/100.

Ten µl of each concentration was added to pairs of monolayers and controls set up.

Control (a)	60 µl haemocytes in GIM	
	10 µl isolate 2417 spores	C-Dox replaces filtrate
	10 µl C Dox medium	
Control (b)	60 µl haemocytes in GIM	
	10 µl isolate 2417 spores	
	10 µl GIM	GIM replaces filtrate
Control (c)	60 µl haemocytes in GIM	
	10 µl GIM	
	10 µl culture filtrate of isolate 304	GIM replaces spores
Control (d)	60 µl haemolymph	
	10 µl GIM	GIM replaces spores
	10 µl GIM	GIM replaces filtrate

The prepared monolayers were incubated at 21° C ± 1° C and 100 % RH for 4 hrs after which the total haemocyte count and number of nodules in 10 microscope fields at × 400 under phase contrast were counted. Paired wells were used for each dilution and the work replicated 3 times (n = 6).

4.2.15 Effect of eicosanoids on haemocyte aggregation

Miller, Nguyen and Stanley-Samuelson (1994) proposed that nodule formation by insect haemocytes was regulated by eicosanoids. These 20-carbon molecules derived from arachidonate have local effects on cells and alter their activities.

The haemocyte aggregation response to bacterial infection in *M. sexta* larvae was reduced after the injection of dexamethasone, or indomethacin. By contrast, when polyunsaturated fatty acids, like arachidonic acid, were injected into infected insects the effect of eicosanoids, like dexamethasone and indomethacin, was reversed and normal haemocyte nodule formation recommenced.

Trials were set up to determine whether dexamethasone and indomethacin affected nodule formation of *M. sexta* haemocytes *in vitro* and whether arachidonic acid could reverse the effects. Combinations of dexamethasone, indomethacin, arachidonic acid, conidiospores and filtrates were investigated to determine which combinations would cause nodulation and which would prevent it.

Twentysix milligrammes of dexamethasone and indomethacin (Sigma) were each dissolved in 10 ml of 95% ethanol. The solutions were then diluted to 1/10 in GIM. Spores of isolate 2417 were prepared in GIM (See section 3.2.3) with a final concentration of 3×10^5 spores per ml. Monolayers and culture filtrate of isolate 304 were prepared as in 4.2.3.

Spores of isolate 2417, which caused nodule formation, were added to the first series of experiments with eicosanoids together with filtrate, dexamethasone or indomethacin as below:-

60µl haemocytes in GIM

10µl spores 2417 at 3×10^5 per ml

10µl culture filtrate of isolate 304, or 10µl dexamethasone, or 10µl indomethacin.

In the control, 10 µl GIM replaced the filtrate or eicosanoids. The monolayers were incubated at 22° C ± 1° C and 100% RH for 4 hours. The total haemocytes and number of nodules in ten microscope fields at × 400 were then counted. Each trial contained 2 monolayers and was replicated three times (n = 6).

The second series looked at the effect of eicosanoids on monolayers without spores and on the combined effects of eicosanoids and culture filtrate of isolate 304 on nodule formation. Monolayers were prepared as before but, apart from the control, without spores.

Monolayers were set up as follows :-

a) 60 µl haemocytes in GIM

10 µl culture filtrate of isolate 304, or indomethacin, or dexamethasone

10 µl GIM

(Effect of individual eicosanoid on haemocytes).

b) 60 µl haemocytes in GIM

10 µl filtrate + 10 µl indomethacin

or 10 µl dexamethasone

or 10 µl additional culture filtrate of isolate 304

(Effect of the combination of filtrate and eicosanoid on the haemocytes and effect of increasing concentration of filtrate).

c) Control (1)

60 µl haemocytes in GIM

20 µl GIM

(Establishes base number of nodules with untreated haemocytes).

d) Control (2)

60 µl haemocytes in GIM

10 µl spores of isolate 2417

10 µl GIM

(Establishes effect of 2417 conidiospores).

The third series of tests measured the effect of arachidonate on monolayers and its ability to aid haemocytes to form nodules after treatment with culture filtrate, dexamethasone or indomethacin.

Miller *et al* (1994) injected 50 µg of arachidonic acid acid in 10 µl ethanol into *M. sexta* fifth instar larvae. Since 10 µl of a 1/10 dilution of eicosanoids in GIM had been effective on the monolayers, similar concentrations and quantities were used for the *in vitro* work with arachidonic acid.

Monolayers were set up as follows:-

i) 60 µl haemocytes — Shows effect of a. a. on haemocytes
10 µl GIM
10 µl arachidonic acid

ii) 60 µl haemocytes — Shows effect of a. a. on filtrate- treated
10 µl culture filtrate of isolate 304 — haemocytes
10 µl arachidonic acid

iii) 60 µl haemocytes — Shows effect of a. a. on haemocytes treated
10 µl spores of isolate 2417 — with spores
10 µl arachidonic acid

iv) 60 µl haemocytes — Control - effect of spores on haemocytes
10 µl spores from isolate 2417
10 µl GIM

v) 60 µl haemocytes — Control - effect of filtrate and spores on
10 µl spores of isolate 2417 — haemocytes
10 µl culture filtrate from isolate 304

vi)	60 µl haemocytes 20 µl GIM	Control - nodule formation by untreated haemocytes

The fourth series compared the combined effect of arachidonic acid and dexamethasone, indomethacin, or filtrate on monolayers with and without spores.

Monolayers were set up as below:-

60 µl haemocytes in GIM

10 µl spores of isolate 2417 at 2×10^5 per ml

10 µl arachidonic acid

10 µl culture filtrate from isolate 304, or indomethacin, or dexamethasone.

The effect of spores on the nodule formation was investigated by setting up a similar series of monolayers, but replacing the spores with GIM.

Controls were set up where arachidonic acid, culture filtrate from isolate 304, indomethacin, dexamethasone were each replaced with 10 µl GIM. The prepared monolayers were incubated at 21° C ± 1° C and 100% RH for 4 hours. Nodules and total haemocytes were counted in ten fields at × 400 under phase contrast.

4.2.16 Assays on the composition of culture filtrate from isolate 304

Culture filtrate from isolate 304 appeared to prevent the nodule formation of haemocytes challenged with isolate 2417 conidiospores (see Table 36). A series of tests was carried out to determine the type of molecule involved.

Monolayers of haemocytes inoculated with isolate 2417 conidiospores were used to test the effect of heating and protease on the culture filtrate of isolate 304. Additionally this filtrate was subjected to membrane filtration in an attempt to determine the molecular size of the active component.

a) Effect of heating filtrate 304

Monolayers of haemocytes in GIM were set up as in 4.2.3 and 10 µl of conidiospores of isolate 2417 (2×10^5 per ml) were added to each monolayer.

Five ml of culture filtrate of isolate 304 was divided into 2 equal aliquots and one was heated to 100° C for 20 min in a water bath and then cooled to 22° C. Ten microlitres of the heated filtrate was added to the experimental monolayers and 10 µl of unheated filtrate to the controls. A control of Czapek-Dox, previously heated to 120° C for 20 min was also included.

Monolayers were incubated at 22° C ± 1° C and 100% RH for 4 hr. Total haemocyte count and number of nodules in 10 fields at × 400 under phase contrast were counted. Six monolayers were prepared for each assay and the work was repeated 3 times (n = 18).

b) Effect of ultrafiltration

To determine the molecular size of the nodule-preventing portion of the culture filtrate of isolate 304, it was passed through centrifugal filter devices (Millipore Corporation, Bedford, MA.), with membranes with cut-off points of 5 kDa and 10 kDa.

Culture filtrate from isolate 304 was thawed from -20° C, aliquots were put in the 5 kDa filter device and centrifuged for 20 min at 1400 *g*. The filtrate was then removed and the portion remaining above the membrane washed with sterile distilled water and centrifuged at 1400 *g* for a further 20 min. This fraction of the filtrate was then placed in a 10 kDa filter device and the procedure repeated, in order to trap molecules larger than 5 kDa and smaller than 10 kDa.

Monolayers of haemocytes were set up (as in 4.2.3) with 10 µl of conidiospores of isolate 2417 at 2×10^5 spores per ml in GIM. Ten µl of the different

culture filtrate fractions were added to each monolayer which were then incubated for 4 hr at 22° C ± 1° C and 100% RH.

After incubation the monolayers were examined at × 400 magnification under phase contrast for total haemocyte count and number of nodules in 10 fields.

Each fraction of culture filtrate was used on a pair of haemocyte monolayers and each essay was repeated 3 times (n = 6).

c) Effect of protease on filtrate 304

The active component of fungal filtrates which affects the nodule-forming response of haemocytes may be a protein. If the filtrate is treated with a wide-spectrum protease any proteins present will be hydrolysed and the treated filtrate will no longer affect nodulation.

Culture filtrate of isolate 304 had passed through a 10 kDa membrane filter was then diluted 1:1 with a solution of a non-specific protease from *Streptomyces griseus* (Product P5147,Sigma). The solution of protease was made up of 100 mg in 100 ml phosphate buffer solution at pH 7.2

The filtrate/protease solution was incubated at 37° C for 30 min. and heated at 100°C for 20 minutes to inactivate the protease. Subsequently it was passed through a 10 kDa membrane device (Millipore Corp.) and centrifuged for 20 min to trap any molecules larger than 10 kDa, which might have remained from the protease treatment.

Monolayers of haemocytes in GIM were set up as before and 10 µl of spores of isolate 2417 (2×10^5 spores per ml) were added.

Control wells contained:-

(i) 10 µl of culture filtrate of isolate 304 diluted 1:1 with sterile PBS,

(ii) 10 µl of liquid C-Dox diluted 1:1 with sterile PBS

Controls of the culture filtrate with the added protease, which had passed through a 10 kDa membrane, were included.

Monolayers were incubated for 4 hr at 22° C ± 1° C and 100% RH. Total haemocytes and number of nodules in 10 fields at × 400 under phase contrast were counted.

d) Quantification of proteins in culture filtrates

The effect of protease on the activity of the fungal culture filtrates towards haemocyte nodulation suggested the presence of small proteins or peptides, since the effect of the filtrates was reduced after protease treatment.

The standard Coomassie Blue G assay (Bradford 1976) was used to determine protein levels in the culture filtrates of isolates 304 and 2417 which had been passed through a 10 kDa membrane and then heated to 100 °C for 20 min.

Dilution series of 1:5, 1:10, 1:25 and 1:50 were prepared from culture filtrates of isolates 304 and 2417 with dH_2O as the diluent. The test was set up in 1.5 ml microcentrifuge tubes with:—

20 µl diluted culture filtrate

50 µl NaOH 1M

1 ml Coomassie Blue G

The Coomassie Blue G dye (Sigma) was made up with:—

0.1% (w/v) Brilliant Blue G

25% (v/v) methanol

5% (v/v) acetic acid

to 1 litre with dH_2O

The microcentrifuge tubes containing the culture filtrate dilutions of isolates 304 and 2417 and reagents were gently inverted and then incubated for 5 min at 22°C.

Two hundred microlitres from each sample was put into wells of a microtitre plate. A blank of dH_2O was included and the samples read on a Dynatech MR5000 plate reader at 595 nm.

The assay was repeated three times. The readings were compared with a calibration curve for protein concentration (bovine serum albumin) and the concentrations in the filtrates calculated and the results expressed in µg/ml.

4.2.17 Gel electrophoresis of treated filtrates

The Bradford assay for proteins (Bradford,1976) showed that there was variation in the amount of protein present in the culture filtrates of isolates 304 and 2417. Gel electrophoresis might show whether this was due to concentrations of the same protein or the presence of proteins in some isolates and not others.

Samples were prepared by passing fungal filtrates from the culture of different isolates through a 10 kDa membrane filter device and then heating them at 100° C for 20 min. Heating did not denature the fraction which appeared to inhibit cell aggregation,but it did denature some of the other proteins in the sample,thus simplifying the identifiaction of the active fraction. Since only small amounts of protein were present treated filtrates were concentrated by drying for 18 hr using a Speed Vac concentrator (Savant) with an Edwards Freeze Dryer Modulo. The dried residues were then re-suspended in sterile distilled water to give final concentrations of 30, 20, 15, and 10 times the original filtrate concentration, after the addition of loading buffer.

SDS gel-loading buffer

50 mM	Tris/HCl (pH 6.8)	6.25ml
100 mM	dithiothreitol	5.0 ml
2%	SDS	23 ml
0.1%	bromphenol blue	2.5 ml
10%	glycerol	8.0 ml
	distilled water	to 100 ml

The dithiothreitol was added just before use and the loading buffer stored at ambient temperature.

After the addition of loading buffer, the samples and the marker were heat shocked for 3 minutes at 100° C and then plunged into ice. Twenty microlitres of each treated sample were loaded into the wells of the stacking gel.

The broad range SDS-PAGE molecular marker was obtained from Bio-rad and contained the following proteins:-

Protein	Daltons
Myosin	200,000
β-galactosidase	116,250
Phophorylase B	97,400
Serum albumin	66,200
Ovalbumin	45,000
Carbonic anhydrase	31,000
Trypsin inhibitor	21,500
Lysozyme	14,400
Aprotinin	6,500

A 5% stacking gel was prepared with the following component volumes:-

distilled water	2.7	ml
30 % acrylamide mix	0.67	ml
1.0 M Tris (pH6.8)	0.5	ml
10 % SDS	0.04	ml
10 % ammonium persulphate	0.04	ml
TEMED	0.004	ml

The proteins in the treated filtrates were known to be less than 10 kDa so a 15% resolving gel, which would reduce the rate of travel of the proteins was used.

15 % Resolving Gel

distilled water	2.3	ml
30 % acrylamide mix	5.0	ml
1.5 M Tris (pH8.8)	2.5	ml
10 % SDS	0.1	ml
10 % ammonium persulphate	0.1	ml
TEMED	0.004	ml

The buffer used for the reservoirs was made up as a 10 times stock solution and diluted when needed. It was cooled on ice before use.

Tris-glycine electrophoresis buffer (10 times)

Tris base	30.3	g
Glycine	144	g
SDS	10	g
Distilled water	1	l

Pairs of gels were run on a Biorad Mini Protean II cell at 120 mA for approximately 1 hour.

After electrophoresis, one gel was fixed and stained with Coomassie blue for 16 hours on a rocking platform and the other fixed and silver stained.

Coomassie blue stain

0.5 % Coomassie Brilliant Blue R-250

45 % methanol

45 % distilled water

9.5 % glacial acetic acid

This gel was then destained for 1 hour with 4 changes of de-stainer :-

45 % methanol

45 % distilled water

10 % glacial acetic acid

A second gel was fixed overnight in:-

44 % methanol

44 % distilled water

12 % acetic acid

with 0.5 ml of 37 % formaldehyde added per litre

Silver staining

The second gel was stained following the method of Hames and Rickwood (1990). It was then washed three times with 50 % ethanol and soaked for 1 min to sensitise it in a solution of 0.04 g of sodium thiosulphate in 200 ml of distilled water. It was then rinsed briefly three times with distilled water and soaked for 20 min in a solution of 200 mg silver nitrate in 200 ml of distilled water with 150 µl of 37 % formaldehyde added just before use.

The gel was rinsed twice with distilled water and developed in a solution of 1.2g of sodium carbonate in 200 ml of distilled water with 10 µl of 37 % formaldehyde and 4 µl of sodium thiosulphate solution (2g in 100 ml). As soon as the bands of marker protein were adequately stained the development was stopped with a solution of :-

44 % methanol

44 % distilled water

12 % acetic acid

The stained gels were then photographed and stored in sterile distilled water with 20 % glycerol at 4° C. Four replicate pairs of gels were run and stained in this way.

Statistics

Data are given as the mean ± standard error (SEM). Statistical significance was calculated with one-way analysis of variance and significant results are shown at either $p<0.05$ or $p<0.01$ level. Regressions and correlations were performed using a standard Minitab package.

4.3 Results

4.3.1 Antibody staining of *M. sexta* larval haemocytes

Antibodies to *Galleria mellonella* and *Manduca sexta* haemocytes were used to stain monolayers of *M. sexta* larval cells (Table 18). Wright's stain was used as a comparison since it differentially stained granular cells and plasmatocytes.

The three antibodies to *M. sexta* haemocytes stained different groups of cells:-

a) Ms13IgG2b stained plasmatocytes (PL) pale brown; the granules of granular cells (GR) bright yellow and dark brown, but did not stain the oenocytoids (O),

b) Ms2IgG1 stained GR and O but not PL, (Plate 7, below)

c) Ms31IgG2b stained O dark brown, but not other cell types.

Four different antibodies to *Galleria mellonella* all stained granular cells and oenocytoids brown. One antibody (GM5) also stained plasmatocytes, whilst another (26B7D8) stained the nucleus of plasmatocytes, but not the cytoplasm. The work showed that antibodies to *Galleria mellonella* larval haemocytes were able to stain haemocytes of *Manduca sexta*.

Plate 7. *M. sexta* haemocytes stained with Ms2IgG1 antibodies

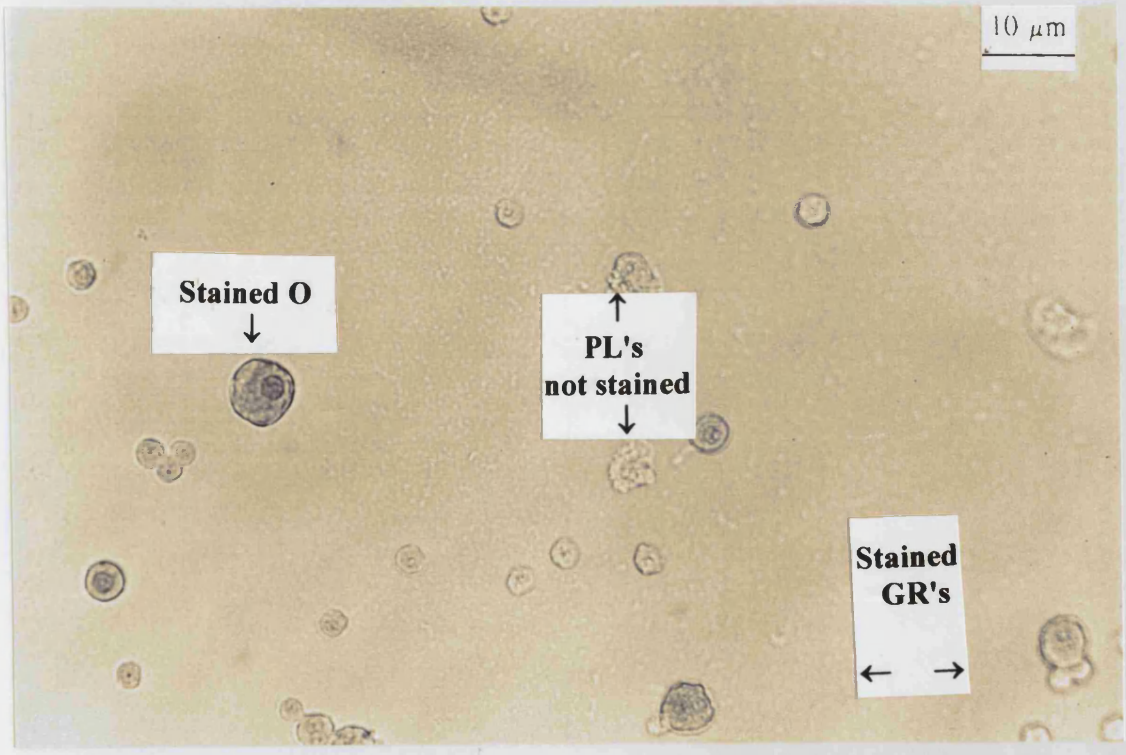


Table 18.

Antibody staining of *M. sexta* haemocytes

	Antibodies to *M. sexta*			Antibodies to *Galleria mellonella*			
Antibody	Ms13 IgG2b	Ms2 IgG1	Ms31 IgG2b	GM2 D1	GM5	20E4 F4H8	26B7 D8M
PL stained	+	-	-	-	+	-	-
GR stained	+	+	-	+	+	+	+
O stained	-	+	+	+	+	+	+

(Three monolayers were stained with each antibody and the responses were uniform i.e. the same cells stained each time)

Monolayers of first day fifth instar *M. sexta* larval cells were prepared by chilling the insects at 4° C for 5 minutes prior to wiping the horn area with 70% ethanol and removing the horn with flamed scissors. The haemolymph was allowed to drip into microcentrifuge tubes containing 30µl of anti-coagulant buffer (see Appendix) at 4° C. The tubes were gently inverted to mix the haemocytes with the buffer and then centrifuged at *77g* for 5 minutes. The supernatant was removed and the haemocytes re-suspended in Grace's Insect Medium.

The cell suspensions were placed on cavity slides and incubated at 25° C ± 1° C in an humidity chamber prior to fixing with 4% formaldehyde for 30 minutes.

Endogenous peroxidases were destroyed by incubating the cells with hydrogen peroxide. The cells were then blocked with a solution of glycine, foetal calf serum, goat serum, Tween 20 and Triton and incubated for 30 minutes.

The blocking reagents were removed and solutions of the different antibodies added to the monolayers which were further incubated for 1 hour at 22° C. The monolayers were then washed three times with PBS and a secondary antibody applied (1 in 500 dilution of goat anti-mouse labelled with

peroxidase enzyme). The secondary antibody was used since it is able to stick to the primary antibody and enhance the response.

After 1 hour's incubation the secondary antibody was removed, the monolayer washed 3 times with PBS and then stained with a 1:1 solution of DABS and hydrogen peroxide for 15 minutes.

The DABS staining was halted by rinsing the monolayers with PBS. They were then examined with an Olympus microscope at × 400.

Wrights stain was used on control monolayers to confirm that no classes of haemocytes had been lost during anti-body staining. Monolayers were fixed with 100% methanol for five minutes and flooded with a 1:4 solution of Wright's stain in PBS for 90 seconds. The stained monolayers were rinsed with PBS and the cells compared with those stained by antibodies.

With Wright's stain, granular cells and oenocytoids stained blue whilst plasmatocytes stained pink with a darker red nucleus. The classes of haemocytes were in similar proportions to those stained with antibodies, confirming that no group of cells had been lost during immunostaining.

4.3.2 Antibody staining of haemocytes from infected insects

Although antibody staining was primarily used to distinguish between different cell types, differences occurred between the staining of cells from infected and uninfected larvae (Tables 19 and 20).

Manduca sexta antibodies

Table 19 Haemocytes from infected larvae stained with *M. sexta* antibodies

	Cell type	Ms13IgG2b	Ms2IgG1	Ms31IgG2b
Injected larvae	PL	+/-	+	+
Injected larvae	GR	+/-	+	+
Injected larvae	O	+	+	+
Immersed larvae	PL	+/-	-	+
Immersed larvae	GR	+	+	+
Immersed larvae	O	+	+	+

Six first day fifth instar larvae were injected with 50µl of 3.7 $\times 10^{6}$ spores per ml of isolate 2727, suspended in sterile Grace's Insect Medium. The control larvae were injected with sterile GIM. The insects were then incubated for 4 hours at 24° C ± 1° C and 100% humidity.

A second group of six first day fifth instar larvae were immersed in the same spore suspension for 10 seconds (the control larvae immersed in sterile GIM for 10 seconds), prior to incubation with the same conditions as the first group, for 72 hours.

After incubation, the haemolymph was removed, centrifuged at 77*g* for 5 minutes and the haemocytes resuspended in GIM. They were then placed on cavity slides, fixed with 4% formaldehyde and stained with the antibodies to either *M. sexta* or *G. mellonella.*

The stained cells were examined with an Olympus microscope at × 400. The work was repeated 3 times.

Plasmatocytes from injected larvae stained with all three *M. sexta* antibodies though plasmatocytes from naive larvae only stained with one (Ms13IgG26). Plasmatocytes from immersed larvae did not stain with antibody Ms2IgG1, but otherwise were the same as injected larvae.

Granular cells and oenocytoids from infected insects stained regardless of the method of infection. This gave a slightly different pattern from the control where oenocytoids did not stain with Ms131bB26 and granular cells did not stain with Ms11gG2b.

Thus, infection with *B. bassiana* conidiospores showed that there were some differences in the ability of *M. sexta* antibodies to stain haemocytes from control and infected insects, and that the mode of infection (immersion or injection) could affect this.

Galleria mellonella antibodies

Table 20 shows the effect of infection on the staining of haemocytes with *Galleria mellonella* antibodies.

Seven first day fifth instar larvae were each injected with 50µl of 3.7×10^6 spores per ml of isolate 2727 suspended in Grace's Insect Medium and then incubated for 4 hours at 24°C ± 1°C. A second group of seven larvae were immersed for 10 seconds in the same spore suspension prior to 72 hours incubation at 24°C.

After incubation the larvae were wiped with 70% ethanol, the horn removed and the haemolymph collected in microcentrifuge tubes containing 30µl of anti-coagulant buffer at 4°C. The tubes were centrifuged at 77*g* for 5 minutes, the supernatant removed and replaced with GIM at 4°C.

The haemocytes were gently re-suspended and allowed to settle on cavity slides for 20 minutes. They were then fixed for 30 minutes with 4% formaldehyde and subsequently washed 3 times with PBS. The monolayers were blocked with 50µl of a solution of 3% foetal calf serum, 100mM glycine, 10% goat serum, 0.05% Tween and 1% Triton 100 and incubated for 30 minutes at 22°C. After incubation the blocking solution was removed and 50µl of the different anti-bodies to *Galleria mellonella* (with blocking reagents, but without Triton) were applied to separate monolayers and incubated for 1 hour at 22°C.

The antibody solutions were removed and the monolayers washed 3 times with PBS. Fifty microlitres of the secondary antibody (1 in 500 dilution of goat anti-mouse antibody labelled with peroxidase enzyme in blocking buffer) was added to each monolayer. The cells were then incubated for 1 hour at 22°C, the secondary antibody removed and the monolayers washed 3 times with PBS.

The cells were then stained with 50µl of 1:1 DABS:hydrogen peroxide solution for 15 minutes and the staining stopped by rinsing with PBS. The monolayers were finally examined with an Olympus microscope at × 400 magnification and the differential staining of cell types recorded.

Controls were made using Wright's stain and the work repeated 3 times for each antibody and method of infection.

Table 20. Haemocytes from infected larvae stained with *G. mellonella* antibodies

Treatment ↓	Haemocyte type ↓	Antibody Gm2D1	Antibody Gm5	Antibody 20E4F4H8	Antibody 26B7D8M
Larvae injected with 50µl of 3.7×10^6 spores of isolate 2727	PL	-	+	-	-
	GR	+	+	+	+
	O	+	+	+	+
Larvae immersed for 10s in 3.7×10^6 spores of isolate 2727	PL	-	-	-	-
	GR	+	+	+	+
	O	+	+	+	+

Table 20 shows that the four different antibodies to *Galleria mellonella* stained granular cells and oenocytoids from infected larvae in a similar way to those from uninfected larvae . However, plasmatocytes from larvae infected by immersion did not stain with Gm5. Infection with *B. bassiana* conidiospores had less effect on haemocyte staining with *G. mellonella* antibodies than it did with staining with the *M.sexta* antibodies.

4.3.3 Germination of spores on haemocyte monolayers

Earlier in this study a considerable difference in the pathogenicity of the different *B. bassiana* isolates for *M. sexta* larvae became evident. Although the ability to penetrate the insect cuticle was shown not to be the cause of this variation in pathogenicity, it was possible that conidiospores of some isolates were unable to germinate in the larval haemolymph. Possibly haemocytes produced, or carried on them, metabolites which could inhibit spore germination and spores of some isolates might be more susceptible than others.

1) The main object of this experiment was to determine if the conidia of twelve *B. bassiana* isolates were able to germinate on haemocyte monolayers and whether there was a difference in the rate of germination between isolates.

Speed of germination (as shown in Table 21) varied between the isolates, but at 8 hours none had more than 4% of germinated spores and at 16 hours three isolates (1315, 1558 and 2417) had more than 20% germinated spores. Germination of the conidiospores on monolayers of haemocytes showed that after 24 hours of incubation there was a considerable difference between isolates. By 48 hours the differences between the isolates had become more marked with two isolates (1315 and 2417) achieving over 90% germination, whilst others (304, 1007 and 1886) still had less than 20% germination.

2) Although the germination trials extended to 48 hours, all the work on cellular responses was carried out after 4 hours incubation, when the responses were clear and not confused by mycelial growth. It is possible that this variation in the rate of germination affects the subsequent development of the fungus and also the insect's ability to contain it.

This disparity between the speed of germination of isolates might be important for subsequent tests involving long incubation periods where germinating spores could affect the response of haemocytes. However, experimental monolayers were incubated for less than 8 hours, at which time even the quickest isolates had only achieved 4% germination.

Table 21. Percentage germination of conidiospores of *B. bassiana* on monolayers of *M.sexta* haemocytes

Isolate	0 h	8 h	16 h	24 h	32 h	40 h	48 h
304	0.17 (0.16)	0.63 (0.33)	0 (0)	1.25 (0.27)	1.31 (0.52)	18.55 (3.3)	20.05 (4.15)
959	0.25 (0.14)	0.46 (5.77)	2.5 (0.9)	2.81 (0.29)	5.7 (2.8)	17.9 (3.7)	19 (2.2)
1007	0.59 (0.16)	0.79 (0.43)	4.8 (0.54)	11.5 (3.0)	10.41 (1.8)	18.05 (3.05)	25.85 (8.45
1122	0.15 (0.13)	0.31 (0.18)	6.85 (1.5)	15.81 (3.9)	20.4 (1.0)	69.65 (0.25)	58.45 (10.45)
1315	1.59 (1.3)	0.16 (0.16)	28.5 (9.8)	89.4 (6.7)	89.7 (1.8)	88.95 (7.85)	97.1 (0.9)
1484	0.60 (0.59)	0.44 (0.42)	2.0 (1.73)	6.6 (10.95)	11.9 (6.7)	11.0 (0.45)	40.95 (0.55)
1558	0 (0)	0.32 (0.32)	23.65 (0.23)	n/a	36.8 (2.7)	36.5 (4.2)	58.9 (3.7)
1629	0 (0)	0 (0)	4.35 (0.1)	18.2 (2.2)	40.3 (2.7)	21.9 (1.4)	30.65 (3.2)
1886	0 (0)	2.03 (0.54)	4.13 (0.6)	8.6 (1.15)	13.9 (0.75)	23.6 *	17.9 *
2417	0 (0)	2.0 (0.29)	28.7 (1.79)	57.5 (5.25)	93.3 (0.14)	91.45 (3.4)	91.35 (0.85)
2727	0.16 (0.16)	0 (0)	1.12 (0.16)	9.5 (0.40)	21.4 (0.57)	15.7 (3)	17.25 (2.95)
3527	0 (0)	4.0 (1.7)	1.75 (0.58)	5.25 (0.57)	87.2 (0.42)	79.95 (4.85)	74.1 (5.2)

Table 21 shows the effect of larval haemocytes in GIM on the germination of conidiospores of different *Beauveria bassiana* isolates. Germination was tested since this might explain the variation in pathogenicity between isolates.

Monolayers of haemocytes were prepared by wiping insects with 70% ethanol and removing the horns of 12 first day fifth instar larvae. The haemolymph was collected in microcentrifuge tubes each containing 30µl of anti-coagulant buffer. It was then centrifuged at 4° C for 5 minutes at *77g* and the supernatant removed and replaced with Grace's Insect Medium. Monolayers of haemocytes were set up on pre-washed, sterile cavity slides.

Washed conidiospores, suspended in sterile distilled water, were counted with a Neubauer haemocytometer and the concentration adjusted to 5×10^6 spores per ml.

30µl of spore suspension was added to each monolayer and they were incubated at 24° C and 100% RH in an humidity chamber.

At 8 hour intervals the monolayers were examined under an Olympus microscope with phase contrast at × 400.

Two hundred spores were counted on each monolayer and each count of the twelve isolates was replicated three times (except * with only 1 replicate). The results are given as percentage germinated spores with SEM of actual counts in brackets.

At 24 hours isolate 304 had the lowest percentage germination (1.25%) and isolate 2417 the highest (57.5%).

4.3.4 Phagocytosis studies

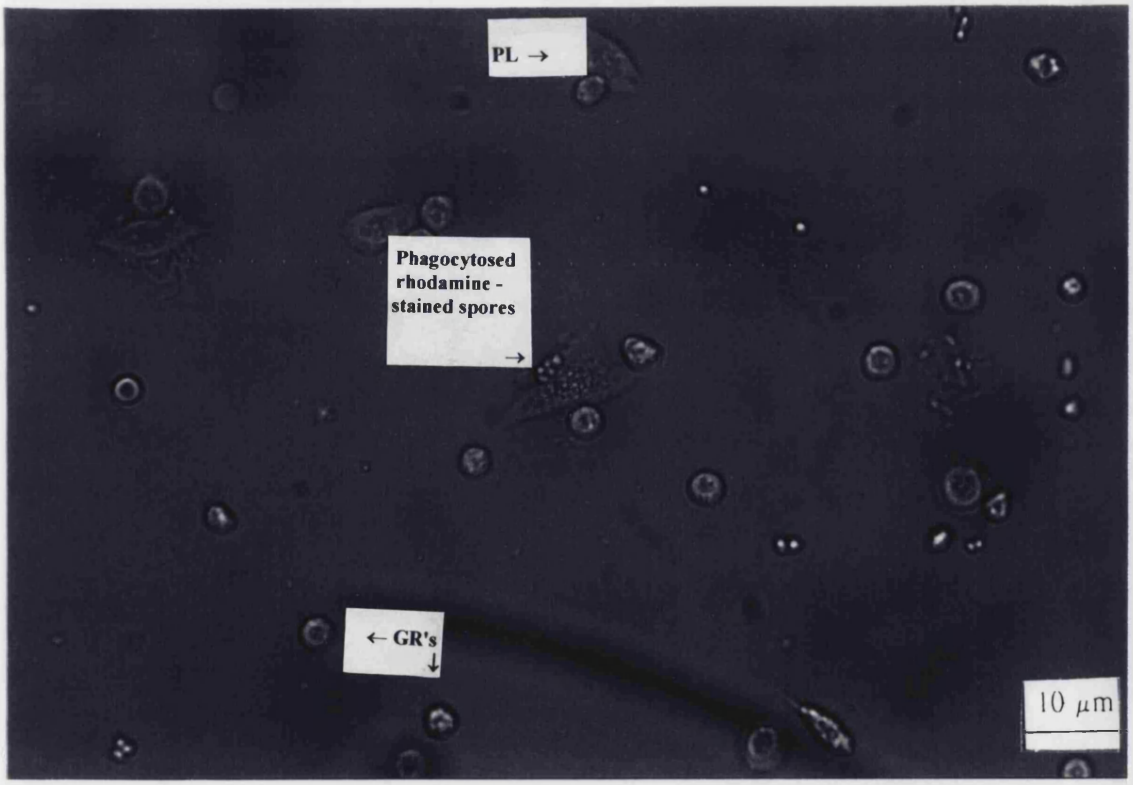
Phagocytosis is frequently assumed to be the major method by which insect haemocytes combat microbial infection. Results from experiments in which monolayers of haemocytes were exposed to added conidiospores showed that only a small percentage of the haemocytes participated in phagocytosis.

Rhodamine-treated conidiospores continued to fluoresce after phagocytosis by haematocytes. The phagocytosed spores were visible within the haemocytes (see Plate 8) and with the method employed it was possible to distinguish between spores which had been ingested and those which adhered to the outside of the haemocytes

Two hundred haemocytes were counted on each of six monolayers and the trials replicated three times. Phagocytosis of fluorescent spores occurred in 1.22% of the haemocytes (SEM 0.1772). Plate 8 was taken under phase-contrast, since with UV light the haemocytes would not be visible.

PLATE 8

Phagocytosis of rhodamine-stained spores by haemocytes



Effect of fungal filtrate on phagocytosis

Where culture filtrate from isolate 304 was added to the monolayers of haemocytes in GIM, the percentage of phagocytosed spores dropped by about half. This difference was statistically significant ($p<0.001$). Although only small numbers of haemocytes ingested conidiospores, the comparison with the control (Table 22) showed that the fungal filtrate affected the ability of the haemocytes to phagocytose.

Table 22

Phagocytosis of *B. bassiana* conidiospores by *M. sexta* haemocytes after 4 hours incubation

Added filtrate 304

Total number of haemocytes	3674
Total number of haemocytes with phagocytosed spores	21
Average % of haemocytes with phagocytosed spores	0.583%
	SEM 0.1679

Control (C-Dox replaces filtrate)

Total number of haemocytes (6 monolayers × 3 replicates)	3620
Total number of haemocytes with phagocytosed spores	44
Average % of haemocytes with phagocytosed spores	1.22%
	SEM 0.1772

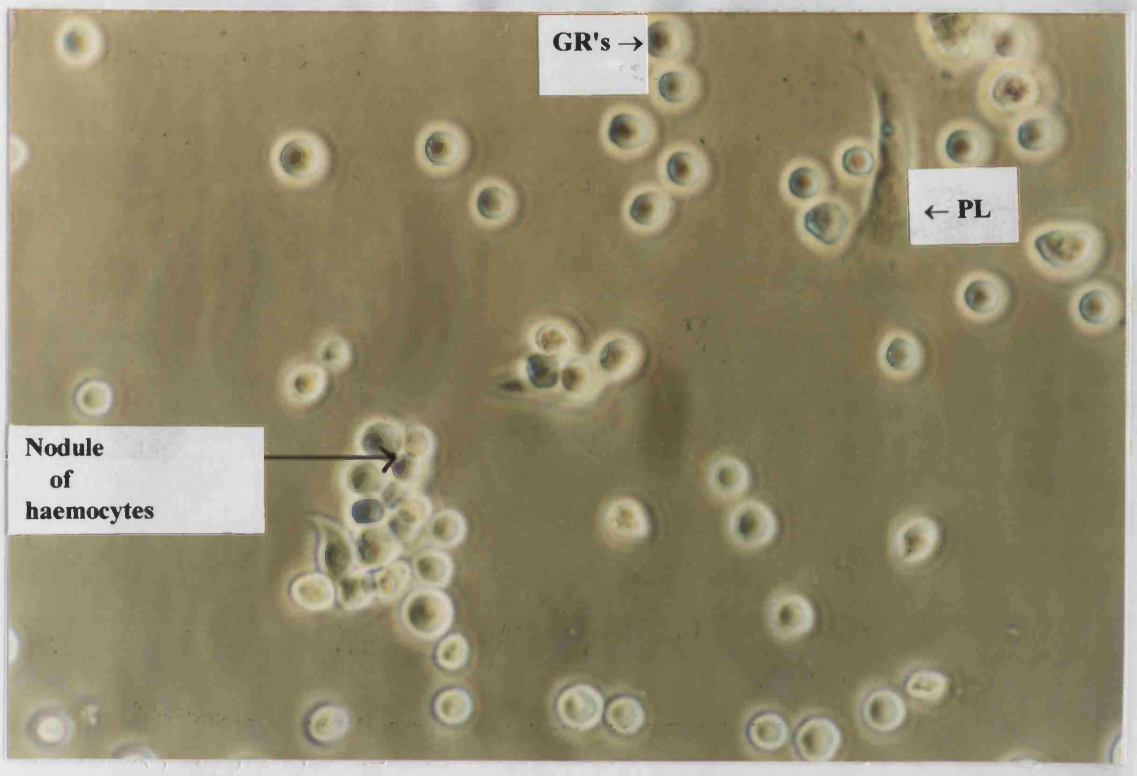
χ^2 values on actual numbers indicated that the number of haemocytes able to phagocytose conidiospores after filtrate treatment were significantly reduced ($p < 0.001$; $\chi^2 = 89.77$ df = 1). One can conclude that the fungal filtrate reduced the phagocytic capabilities of conidiospores.

4.3.5 The effect of conidiospores of different *B. bassiana* isolates on the nodule formation by haemocytes of *M.sexta* larvae

The effect of conidiospores of twelve different isolates on larval haemocytes was compared *in vivo* and *in vitro.*

In vivo

PLATE 9 Early aggregation of larval haemocytes



Fifty microlitres of conidiospores of isolate 2417 at 2 × 10^{6} spores per ml in GIM were injected into first day fifth instar larvae. The larvae were incubated at 23° C ± 1° C for 4 hours. After chilling at 4° C for five minutes they were wiped with 70% ethanol and the horn removed with flamed scissors.

Haemolymph was then collected in micro-centrifuge tubes containing 30µl of anti-coagulant buffer at 4° C. The tubes were centrifuged for 5 minutes at *77g* ,the supernatant removed and replaced with GIM. The spore / haemocyte suspension was put on a cavity slide and examined at × 400 with an Olympus microscope.

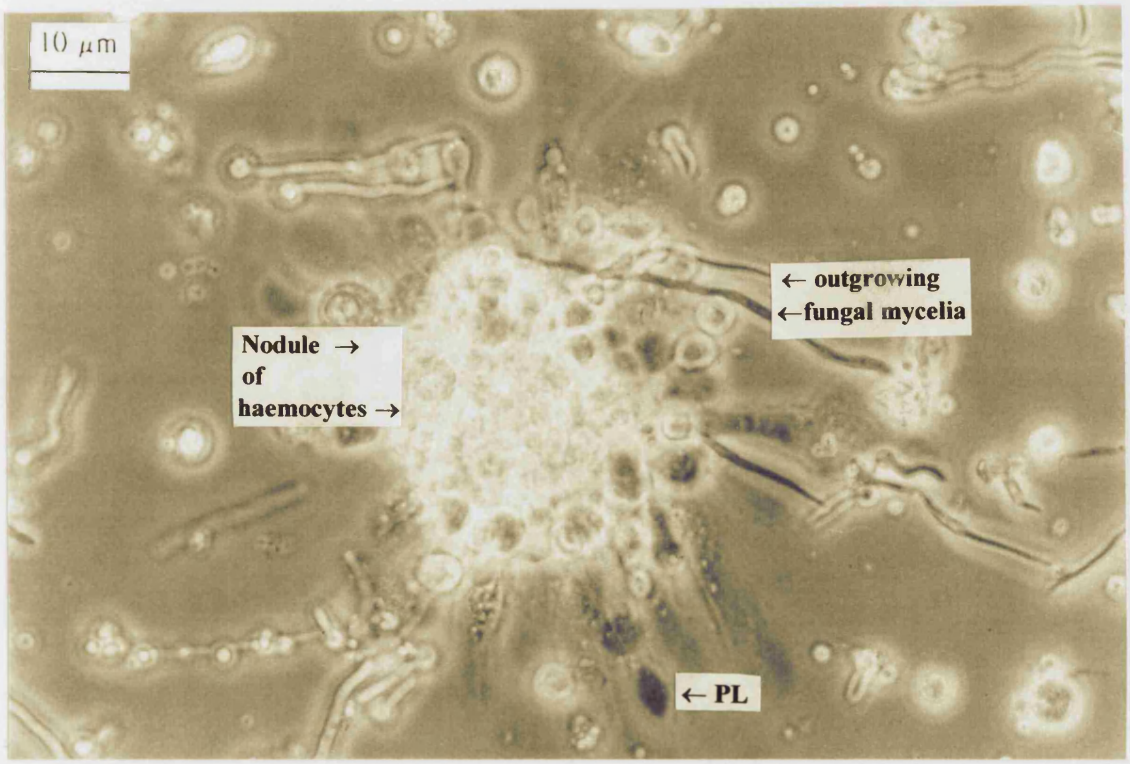
After inoculation with conidiospores larval haemocytes aggregated in a particular sequence. Granular cells formed small nodules which entrapped conidiospores (Plate 9). Gradually a mixed group of cells formed large nodules of more than fifty haemocytes with entangled conidiospores. Groups of haemocytes were counted as nodules when five or more cells were contiguous .

There was considerable difference between the number of nodules formed at 4 hours as a result of injection of spores of different isolates (Table 23). Seven isolates (959,1122,1484,1558,1629,1886 and 2417) were significantly ($p<0.01$) different from the control.

Control haemolymph showed a steady low number of nodules, so that handling and injection of GIM alone did not appear to stimulate nodule production. All of the isolates caused the production of more nodules than the control. Whereas some isolates appeared to provoke the production of few nodules, which did not increase greatly over time, others produced a response that continued to increase during all of the 6 hours of the experiment . Conidiospores of isolate 2417 were notable for stimulating considerably more nodules ($p< 0.01$) than spores of other isolates (see Table 23). Five isolates (1007,1315,1558,1886 and 3527) produced between 12% and 60% more nodules at 6 hours than 4 hours possibly coinciding with a change in the spore surface at this time.

PLATE 10

Aggregation of larval haemocytes 24 hours after inoculation



The cavity slides, which contained haemocytes and conidiospores of isolate 2417 suspended in GIM, were incubated for 24 hours in an humidity chamber (100% RH) at 22° C ± 1° C. They were then re-examined at × 400 with an Olympus microscope

At 24 hours post-inoculation some of the germinated conidiospores had developed mycelia and grown out of the haemocytic nodules which had previously enveloped them (Plate 10).

Table 23 shows the number of nodules formed in the haemolymph of *M. sexta* larvae injected with conidiospores of different *B. bassiana* isolates.

First day fifth instar larvae were injected with 50µl of 2×10^6 spores per ml suspended in GIM. The insects were incubated from 0 to 6 hours at 25°C ± 1°C. They were then chilled on ice and the horn removed with flamed scissors. The haemolymph was allowed to run into 1.5ml microcentrifuge tubes containing 30µl of anti-coagulant buffer at 4°C. Each 1d5L larva yielded approx. 100µl of haemolymph.

The microcentrifuge tubes were centrifuged at 77*g* for 5 minutes then 100µl of supernatant was removed and replaced with 70µl GIM at 4°C, to give approx. the same concentration of haemocytes in haemolymph as *in vivo*.

The suspensions of haemocytes and spores in GIM were placed on cavity slides and examined with an Olympus microscope at × 400 magnification. Groups of haemocytes were counted as nodules when 5 or more were contiguous. Ten microscope fields were counted and the work repeated 3 times for each time point for each isolate.

Variation in the number of nodules formed by haemocytes of insects injected with different isolates is shown in Table 23 and Figures 17a) and 17b). At four hours post-injection seven isolates had stimulated the production of a significant number of nodules ($p < 0.05$), though there was no further significant increase (*in vivo*) at 6 hours.

The difference in LT_{50} between isolates (Table 3) was echoed by the variation in the number of nodules produced by larvae injected with different isolates. Regression of the number of nodules at 4 hours *in vivo* against LT_{50} (Fig 17c) showed a significant correlation ($p < 0.001$). This correlation was, however, strongly dependent on the 4 hour nodulation response to just one isolate (2417).

One-way analysis of variance at 4 hours showed the number of nodules was significant at $p < 0.05$ with 7 isolates and $p < 0.01$ for 4 isolates (starred * in the table).

Table 23

Number of nodules produced *in vivo* by *M. sexta* larvae injected with conidiospores of different *B. bassiana* isolates

Isolate	Time post infection	1 hr	2 hr	4 hr	6hr
304	Mean	2	4.8	4.0	2.5
	SEM	0.81	2.15	1.45	1.5
	p			0.141	
959	Mean	1.33	1.0	7	5
	SEM	0.49	0	1.24	1.06
	p			* 0.0085	
1007	Mean	2.0	3.5	2.33	3.16
	SEM	0.36	0.62	0.49	0.65
	p			0.383	
1122	Mean	2.33	5.35	7.6	6.6
	SEM	1.05	0.98	2.04	1.8
	p			0.020	
1315	Mean	3.7	4.6	4.3	5.76
	SEM	1.27	2.2	1.2	0.77
	p			0.148	
1484	Mean	2.83	4.83	6.83	5.83
	SEM	0.47	0.93	1.7	0.6
	p			0.024	
1558	Mean	4	5.3	6.5	8.7
	SEM	0.44	0.76	0.57	1.59
	p			* 0.0035	
1629	Mean	0.67	2.66	5.33	4.16
	SEM	0.66	0.66	0.616	1.69
	p			* 0.0037	
1886	Mean	n/a	6.66	4.67	5.33
	SEM		1.44	1.05	1.28
	p			0.032	
2417	Mean	3.3	5.9	16.4	16
	SEM	0.73	1.0	1.8	0.63
	p			* 0.0026	
2727	Mean	1.5	2.83	2.66	2.657
	SEM	0.8	0.5	0.47	0.48
	p			0.058	
3527	Mean	3.66	1.83	2.0	5.0
	SEM	0.8	0.47	0.44	0.51
	p			0.54	
Control	Mean	1.1	1.8	1.5	1.9
	SEM	0.37	0.36	0.27	0.34

(n = 6)

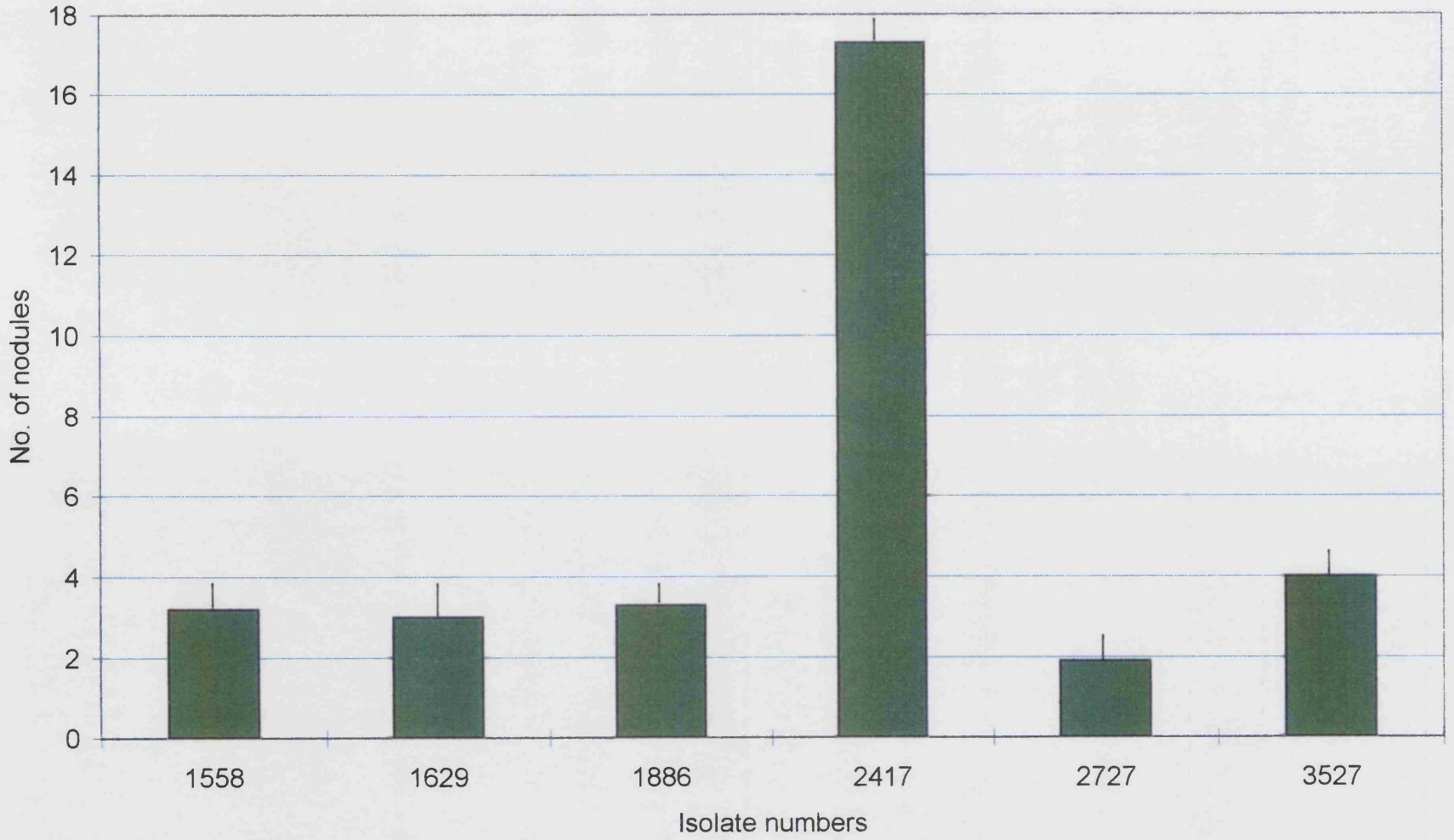
Figures 17a and 17b (overleaf) show the number of haemocyte nodules (10 microscope fields at × 400) formed from the haemolymph of *M. sexta* larvae 4 hours after injection with 50µl of 2×10^6 spores per ml of different isolates of *B. bassiana.*

The number of nodules produced after injection was regressed against the LT50 (2.3.1 Table 3) of each isolate using a Minitab package (Minitab Statistical Software, Pennsylvania, USA). The results (Figure 17c) showed a positive relationship between the number of nodules and the LT50 of the different isolates. The larger the number of nodules the longer the LT50 and conversely the fewer the nodules the shorter the LT50.

R squared (coefficient of determination) is the proportion of variation in y accounted for by variation in x. The larger the value of R^2 the more useful the independent variable is likely to be as a predictor. 95% confidence bands (shown by red dotted line) indicate the zone within which there is 95% confidence that the whole population will lie. 95% predictor bands (dashed blue line) give the confidence zone for individual values.

Fig 17 a

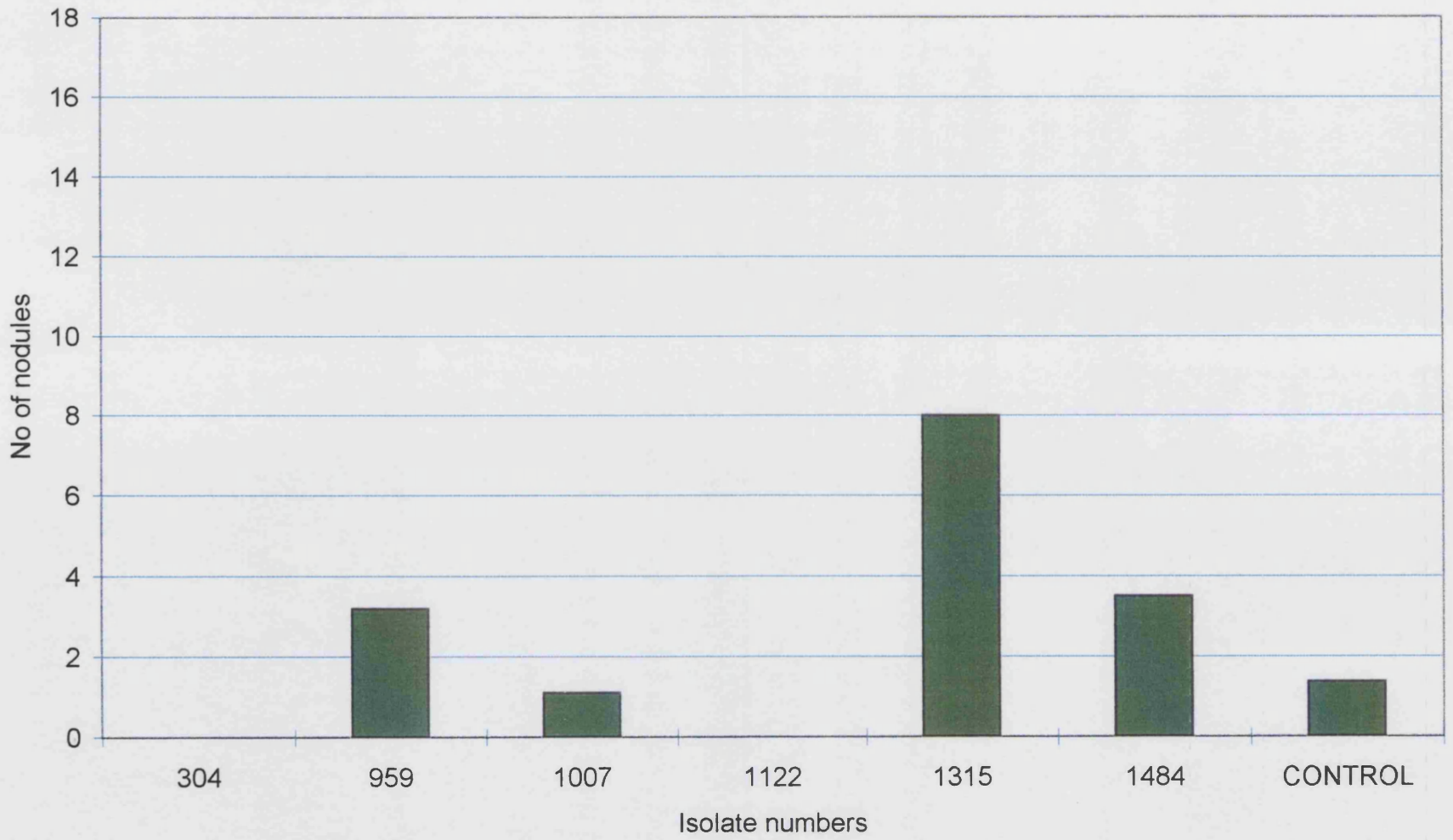
Nodules produced at 4 hr in vivo



Haemocyte groups counted as nodules when 5 or more cells are contiguous
n = 6

Fig 17 B

Nodules produced at 4 hr in vivo



Haemocyte groups counted as nodules when 5 or more cells are contiguous
n = 6

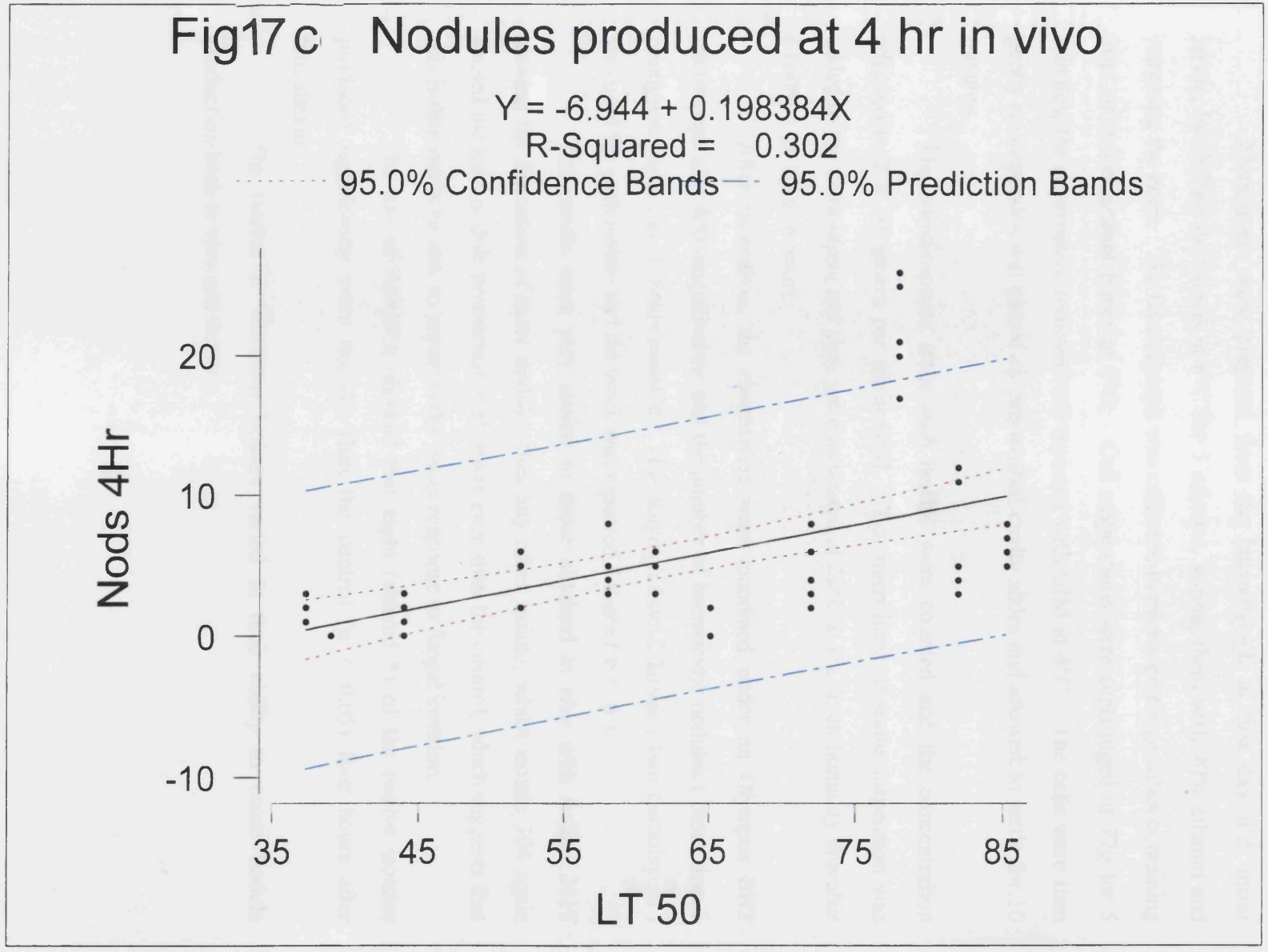


Fig17 c Nodules produced at 4 hr in vivo
Y = -6.944 + 0.198384X
R-Squared = 0.302
95.0% Confidence Bands
95.0% Prediction Bands
Nods 4Hr
20
10
0
-10
35
45
55
65
75
85
LT 50

In vitro

Table 24 compares the effect on monolayers of haemocytes of conidiospores from 12 different *Beauveria bassiana* isolates.

Monolayers were prepared from the haemolymph of first day fifth instar larvae, by chilling the insects at 4°C for 5 minutes, wiping them with 70% ethanol and removing the horn. The haemolymph was collected in microcentrifuge tubes containing 30µl of anti-coagulant buffer at 4°C. Cell suspensions were centrifuged at 77*g* for 5 minutes, the supernatant removed and replaced with GIM at 4°C. The cells were then gently re-suspended and placed on pre-washed cavity slides and allowed to settle for 10 minutes.

The conidiospores from each isolate were counted and the concentration adjusted to 2×10^6 spores per ml in GIM. Ten microlitres of spore suspension was added to the monolayers and they were incubated at 22°C ± 1°C in an humidity chamber (100%) for up to 6 hours.

After incubation, the monolayers were examined under an Olympus BH2 microscope at × 400 magnification and the number of haemocytic nodules (more than 5 contiguous cells) in 10 fields counted. Haemolymph from 2 larvae (two monolayers) was used for each isolate and the work was repeated 3 times (n = 6).

The results were very similar to those obtained *in vivo* with isolate 2417 causing the formation of more nodules than any other isolate, whilst isolate 304 again caused the least nodule production with fewer even than the control, which suggests that this isolate might be able to suppress the insect response to fungal invasion.

The use of ANOVA showed that eight (starred *) of the twelve isolates produced significantly more nodules than the control ($p < 0.05$) four hours after inoculation.

The twelve *B. bassiana* isolates varied in their ability to cause nodule production both *in vivo* and *in vitro*.

Table 24

Number of nodules produced *in vitro* by *M.sexta* haemocytes infected with spores of different *B. bassiana* isolates

Isolate	Time post inoculation	0 hr	1 hr	2 hr	4 hr	6 hr
304	Mean	0	0	0	0	0.3
	SEM	0	0	0	0	0.204
	p				* 0.044	
959	Mean	0.83	0	1.5	4.6	4.16
	SEM	0.48	0	0.62	0.71	0.40
	p				* 0.038	
1007	Mean	0.50	0.83	0.67	1.17	4.16
	SEM	0.49	0.40	0.41	0.48	0.87
	p				0.576	
1122	Mean	0.30	1.67	2.67	1.66	1.16
	SEM	0.33	0.66	0.66	0.32	0.40
	p				0.842	
1315	Mean	1.5	6.3	8.33	6.3	4.8
	SEM	0.73	0.87	1.32	0.80	1.35
	p				* 0.00987	
1484	Mean	0.8	6.3	6.2	7.8	12.8
	SEM	0.31	1.74	1.59	1.76	4.56
	p				* 0.0246	
1558	Mean	0.3	3.7	4.3	4.3	3.5
	SEM	0.2	0.33	0.87	0.61	0.98
	p				* 0.042	
1629	Mean	1.6	0.6	2.3	2.8	2.66
	SEM	0.33	0.21	0.33	0.31	0.33
	p				0.121	
1886	Mean	3.3	4.3	3.8	6.6	8.5
	SEM	1.20	0.66	1.24	0.49	1.58
	p				* 0.00012	
2417	Mean	4.16	13.8	16.6	21.6	19
	SEM	0.73	3.06	2.3	1.34	2.48
	p				* 9.2×10^{-6}	
2727	Mean	0.16	1.0	2.0	2.3	1.6
	SEM	0.16	0.36	0.81	0.33	0.33
	p				0.289	
3527	Mean	1.3	3.3	6.3	5.16	7.16
	SEM	0.61	1.06	0.42	0.65	0.83
	p				0.060	
Control	Mean	0.7	0.76	1.35	1.29	1.23
	SEM	0.21	0.26	0.31	0.32	0.30

(n = 6)

Analysis of variance was used to compare the number of nodules and significantly different results ($p < 0.05$) are starred (*).

4.3.6 Effect of conidial concentration on nodule formation *in vitro*

Different conidial concentrations of isolates 304 and 2417 were added to haemocyte monolayers. The number of nodules formed was assessed at 1, 4, and 6 hours post-treatment.

Isolate 304

Table 25 shows the effect of the conidial concentration of isolate 304 on the formation of haemocytic nodules *in vitro.*

Washed conidiospores were suspended in GIM and counted on a Neubauer haemocytometer. Dilutions of 5×10^7; 5×10^6; 5×10^5 and 5×10^4 were made in GIM. First day fifth instar larvae were chilled at 4°C for 5 minutes, wiped with 70% ethanol and the horn removed with flamed scissors. The haemolymph was collected in microcentrifuge tubes containing 30µl of anti-coagulant buffer at 4°C. The tubes were then centrifuged at 77*g* for 5 minutes, the supernatant removed and replaced with GIM at 4°C.

Ten microlitres of spore suspension was added to monolayers of 90µl of haemocyte suspension and gently mixed. The monolayers were incubated at 22°C ± 1°C and 100%RH for 1 to 6 hours and examined using an Olympus BH2 microscope at × 400 magnification. The number of haemocytic nodules (aggregations of 5 or more cells) in 10 fields was counted and the work repeated six times for each spore concentration.

Table 25 The effect of concentration of Isolate 304 conidiospores on the formation of haemocytic nodules *in vitro*

Incubation Time ↓	Concentration of spores per ml →	10µl of 5×10^7	10µl of 5×10^6	10µl of 5×10^5	10µl of 5×10^4	10µl CDox (Control no spores)
1 h	Mean no. nodule	0.83	0.86	1.29	6.0	1.0
	SEM	0.31	0.28	0.31	1.25	0.33
	p	1.000	1.00	0.525	0.00	
	p*	0.001*	0.001*	0.0026*		
4h	Mean no.nodule	0.83	0.33	1.66	4.83	0.5
	SEM	0.40	0.33	0.56	1.39	0.34
	p	0.541	0.765	0.160	0.013	
	p*	0.039*	0.032*	0.042*		
6h	Mean no.nodule	1.167	0.667	0.33	9.8	1.167
	SEM	0.60	0.42	0.21	1.4	0.54
	p	1.00	0.484	0.183	0.000	
	p*	0.001*	0.0009*	0.0008*		

(n = 6)

An unstacked one-way ANOVA showed the probability (p) of difference between the nodules produced at different concentrations of spores and the control (Czapek-Dox, no spores). Starred figures compare 5×10^4 concentrations of spores with higher concentrations.

Table 25 shows that after 1 hour there was a significant difference between the number of nodules in the control and those produced after incubation with the lowest concentration (5×10^4 spores per ml) of 304 conidiospores ($p < 0.005$). The difference at this concentration was also significant ($p<0.01$) at 4 and 6 hours.

A one-way ANOVA showed that a conidiospore concentration of 5×10^5 spores per ml and above did not differ significantly from the control in its ability to

stimulate nodule production, when different concentrations of spores were compared with each other (starred figures in Table 25). There were significantly more nodules produced after inoculation with the lowest concentration of spores (5×10^4) than with the higher concentrations, suggesting that the higher concentrations of spores may inhibit haemocyte response.

Isolate 2417

Table 26 shows the mean number of nodules produced by monolayers of haemocytes inoculated with isolate 2417 conidiospores. One-way ANOVA was used to compare nodule numbers between the control (no spores) and different concentrations of conidiospores. It showed a highly significant difference in the number of nodules formed in all except one case (5×10^5 spores per ml at 1 hour).

When the higher concentrations were compared with the lowest (5×10^4 spores per ml) a significant difference (p*) was evident after 2 hours incubation. The number of nodules produced in response to the conidiospores was greater at higher concentrations, but dose dependence was not clearly evident. It is possible that a threshold is reached at 5×10^4 spores per ml and that dose dependence would only be evident with smaller intervals between 5×10^4 and 5×10^5 spores per ml.

From this point forward, concentrations of 5×10^5 spores per ml of isolate 2417 were used to inoculate monolayers . They gave a clear result after 4 hours incubation, which was possible to enumerate accurately and was replicable.

Table 26

The effect of concentration of isolate 2417 conidiospores on the formation of haemocyte nodules *in vitro*

Incubation time ↓	Concentration of spores per ml →	10µl 5×10^7	10µl 5×10^6	10µl 5×10^5	10µl 5×10^4	10µlGIM (no spores)
1h	Mean no. nodules	4.16	4.833	1.67	3.67	0.667
	SEM	1.00	1.00	0.803	0.557	0.211
	p	0.000	0.000	0.256	0.000	
	p*	0.517*	1.34*	0.102*		
2h	Mean no. nodules	8.83	9.5	10.66	2.66	1.166
	SEM	0.600	0.562	2.17	0.333	0.306
	p	0.000	0.000	0.000	0.000	
	p*	0.001*	0.000*	0.018*		
4h	Mean no. nodules	16.83	16.167	19.5	6	0.66
	SEM	0.702	0.653	1.41	0.63	0.333
	p	0.000	0.000	0.000	0.001	
	p*	0.001*	0.000*	0.000*		
6h	Mean no. nodules	29.66	29.3	26.27	10.16	1.5
	SEM	0.88	1.115	4.20	1.275	0.223
	p	0.000	0.000	0.000	0.0001	
	p*	0.000*	0.000*	0.016*		

(n = 6)

Table 26 shows the effect on nodule formation of conidiospore concentration of isolate 2417. Four different concentrations of conidiospores of isolate 2417 were made in GIM Ten microlitres of these suspensions were added to 90 µl of first day fifth instar larval haemocytes suspended in GIM. After gentle inversion, they were placed on cavity slides and incubated for 1 to 6 hours at 22°C ± 1°C and 100%RH (preparation as for Table 25).

The monolayers were examined at × 400 magnification and the number of haemocytic nodules (aggregations of 5 or more cells) counted. The work was repeated six times. An unstacked one-way ANOVA was used to compare nodule numbers from inoculated monolayers with the control and its associated probability (p) is shown. p*gives the probability of difference between the lowest spore concentration (5×10^4) and higher concentrations.

4.3.7 Effect of heating conidiospores on nodule formation

Insect haemocytes may be stimulated by non-biological material, like Sephadex beads to produce nodules. Tests were carried out with heat-killed conidiospores to see if they too could stimulate the nodule response of haemocytes.

Autoclaved spores of isolate 304 and 2417 were compared with non-heated spores for their ability to stimulate nodule production both *in vivo* and *in vitro*. These two isolates were chosen since earlier work (4.3.3) showed that they caused extremes of nodule production.

Total haemocytes, plasmatocytes, granular cells and oenocytoids were counted, but when the monolayers were compared there was no significant difference between the numbers of any cell group in any of the treatment groups (Table 27 overleaf).

Table 27

Effect of heating conidiospores on nodule production

	Isolate 2417			
	In vivo		*In vitro*	
	Heated 120°C/20mins	Non-heated	Heated 120°C/20mins	Non-heated
Mean no. of nodules	1.5	15	1.33	19.6
SEM	0.957	1.145	0.617	1.803
	$p < 0.001$		$p<0.001$	

	Isolate 304			
	In vivo		*In vitro*	
	Heated 120°C/20mins	Non-heated	Heated 120°C/20mins	Non-heated
Mean no. of nodules	1.83	1.83	1.16	0.5
SEM	0.401	0.3	0.617	1.803
	$p = 1.00$		$p = 1.00$	

	Control without spores	
	In vivo	*In vitro*
Mean no. of nodules	2.714	1.25
SEM	0.626	0.479

(4h incubation /n = 6)

Table 27 shows the effect of heating conidiospores of isolates 2417 and 304 on the nodule production of larval haemolymph both *in vivo* and *in vitro*.

Conidiospores from both isolates were harvested with 0.05% Tween in sterile distilled water. They were washed 3 times with distilled water and the spores of each

isolate divided into 2 aliquots in sterile glass universals. One aliquot of each isolate was heated to 120°C for 20 minutes and then cooled to 22°C.

All four aliquots (heated and non-heated of both isolates) were counted with a haemocytometer and then diluted in GIM to give 3×10^5 spores per ml.

For *in vivo* trials, 2 first day fifth instar larvae were each injected with 50µl of 3×10^5 spore suspension and incubated for 4 hours at 25°C ± 1°C with 100%RH. The larvae were wiped with 70% ethanol, the horn removed and the haemolymph collected in microcentrifuge tubes containing 30µl of anti-coagulant buffer at 4°C. The tubes were centrifuged at *77g* for five minutes, the supernatant removed and replaced with GIM at 4°C. The microcentrifuge tubes were gently inverted and the haemocyte suspension placed on sterile cavity slides.

Ten fields were examined at × 400 magnification with an Olympus BH2 microscope and groups of five or more haemocytes were counted as nodules.

In vitro

Haemolymph monolayers were prepared as *in vivo.* Ten microlitres of spore suspension at 3×10^5 spores per ml in GIM (heated, or non-heated of isolate 304 or 2417) was added to each monolayer. The monolayers were incubated for 4 hours at 22°C ± 1°C with 100% RH. The number of nodules in ten microscope fields at × 400 magnification were counted.

The work was repeated 3 times (n = 6) both *in vivo* and *in vitro.*

The probability (p) of difference in the effect of heated and non-heated spores was calculated with unstacked one-way ANOVA.

If the spores of Isolate 304 had an immuno-suppressive effect on the haemocytes which was destroyed by heating, the heated spores would cause more nodules than the unheated, whereas heating did not alter the lack of nodules after treatment.

Isolate 2417

There was a highly significant difference ($p < 0.001$) between the number of nodules produced *in vivo* after injection with heated and non-heated spores (cf. Table 27). This was repeated *in vitro* ($p < 0.001$) and showed clearly that heat-treated conidiospores no longer stimulated haemocytes to produce nodules. The effect of nodule induction by spores of this isolate was destroyed by heating.

Isolate 304

Monolayers from larvae treated with heated ,or non-heated spores *in vivo* and *in vitro* did not produce a significantly different number of nodules ($p = 1.00$).

Plates of SDAY which had been streaked with heat-heated conidiospores of both isolate 2417 and 304 showed no growth after 7 days incubation, whilst unheated cultures grew normally.

4.3.8 Effects of culture filtrate from different *B. bassiana* isolates on nodule production by haemocytes.

It has already been shown that nodule formation by haemocytes in response to conidia varies between isolates (4.3.5), both *in vivo* and *in vitro*.

Filtrates from *B. bassiana* cultures grown *in vitro* also showed considerable difference in their effects on haemocytes.

Although the liquid cultures of different isolates had been grown under the same conditions and for the same length of time (see Table 11) the effect of their filtrates on the formation of haemocyte nodules varied considerably. None of the plates streaked with the filtrate of the twelve different isolates showed any microbial growth after 5 days incubation.

Addition of conidiospores and filtrate to monolayers

Conidiospores of isolate 2417 had been shown to promote the formation of haemocyte nodules (Table 24). The culture filtrates from all twelve isolates were tested on haemocyte monolayers treated with isolate 2417 conidiospores to monitor the effect of filtrate on nodule formation. The results are given in Table 28.

Table 28

Comparison of nodule formation by monolayers treated with 2417 spores and filtrates from the growth of different isolates

Filtrate/Isolate	Mean no. of nodules	SEM
304	3.3	0.58
959	5.91	0.908
1007	10	1.02
1122	6.92	0.98
1315	13.25	1.89
1484	11.91	2.81
1558	6.16	1.43
1629	5.69	0.79
1886	3.17	0.82
2417	21.5	2.73
2727	14.2	1.19
3527	12.55	0.82
Control, no spores	4	0.85
Control, no filtrate	18.25	0.84

(n = 12)

Conidiospores from 12 isolates of *B. bassiana* were harvested, washed and suspended in sterile distilled water. They were counted with a Neubauer haemocytometer and the concentration adjusted to 1×10^7 spores per ml.

One ml of spore suspension was inoculated into 100ml of Czapek Dox liquid media in 250ml conical flasks. The flasks were incubated at 25°C on an orbital shaker at 120 rpm for 8 days. The cultures were then filtered through sterile muslin and the filtrate centrifuged at 1407*g* for 20 minutes. Ten ml aliquots of supernatant were filtered through 0.45µm Acrodiscs (Gelman Sciences) to remove any fungal fragments.

SDAY plates inoculated with 50µl of fungal filtrate and incubated for 7 days at 25°C ± 1°C showed no fungal growth.

First day fifth instar larvae were wiped with 70% ethanol and the horn removed with flamed scissors. The haemolymph was collected in microcentrifuge tubes

containing 30µl of anti-coagulant at 4°C. The tubes were gently inverted and then centrifuged at 77*g* for 5 minutes. Supernatant was removed and replaced with Grace's Insect Medium at 4°C. Sixty microlitres of haemocyte suspension were put on each sterile cavity slide with 10µl of isolate 2417 spore suspension at 1.9×10^5 spores per ml and 10µl of culture filtrate added. Liquid Czapek Dox replaced culture filtrate in the control.

Monolayers were incubated in an humidity chamber at 22°C ± 1°C and 100% RH for 4 hours. They were then examined at × 400 magnification with an Olympus BH2 microscope and the number of nodules (5 or more adhering haemocytes) counted in 10 fields.

The work was repeated 3 times with 4 insects for the filtrate of each fungal isolate (n = 12).

Table 28 shows the difference in the number of nodules produced by monolayers of haemocytes with added conidiospores of isolate 2417. Monolayers subsequently treated with the culture filtrates from the different isolates showed that a significant ($p<0.05$) reduction in nodule production occurred after treatment with filtrates from the culture of isolates 304,959,1122,1558, 1629 and 1886.

Since "control,nofiltrate" gives a high nodule count dependent on 2417 spores, any reduction in nodule count is due to the individual culture filtrate.

Effect of culture filtrate of isolate 304 on the nodule formation of haemocytes

Although spores of isolate 2417 stimulated considerable nodule formation, the addition of culture filtrate of isolate 304 reduced this to the level of the control. Table 29 shows the effects on haemocyte monolayers of spores and culture filtrate of isolate 304 separately and together.

Table 29

Comparison of monolayers treated with a combination of 2417 spores and 304 culture filtrate, with 2417 spores or culture filtrate from isolate 304 alone

	10µl 2417 spores with10µl304 culture filtrate	10µl 2417 spores with 10µl Czapek Dox	10µl 304 filtrate with 10µl Czapek Dox	20µl Czapek Dox
Mean no. of nodules	2.4167	29.54	0.166	2.25
SEM	0.350	2.008	0.166	0.4392

(n = 24)

Haemocyte monolayers were prepared by chilling 8 first day fifth instar *M. sexta* larvae, wiping them with 70% ethanol and removing the horn. The haemolymph was collected in microcentrifuge tubes containing 30µl of anti-coagulant buffer at 4°C. These were then centrifuged at 77*g* for 5 minutes after which the supernatant was removed and replaced with GIM at 4°C. The cell suspensions were gently inverted and then placed on sterile cavity slides.

Conidiospores of isolate 2417 were washed, suspended in GIM and counted with a Neubauer haemocytometer. The concentration was adjusted to 1.9×10^5 spores per ml in GIM and 10µl added to the monolayers.

Filtrate from the 8 day growth of isolate 304 was re-filtered through 0.8/0.2µm Acrodiscs (Gelman Sciences) and 10µl added to the monolayers which were incubated for 4 hours at 22°C ± 1°C and 100% RH. Ten microlitres of sterile CDox liquid replaced the filtrate or the spore suspension in the controls.

The monolayers were examined under phase contrast at × 400 with an Olympus BH2 microscope and the number of haemocyte nodules (aggregations of 5 or more cells) in 10 fields was recorded. The work was repeated 3 times (n = 24).

A one-way ANOVA test indicated that the monolayers treated with spores and filtrate versus spores alone were significantly different ($p < 0.001$) . Spores with filtrate were also significantly different from filtrate alone ($p < 0.001$), but when spores and filtrate were compared with the control Czapek Dox the result was not significantly different ($p = 0.278$).

Effect of culture filtrate from isolate 2417 on nodule formation by haemocytes

By contrast with culture filtrate of isolate 304, treatment of monolayers with isolate 2417 spores and isolate 2417 culture filtrate (Table 30) showed that this filtrate did not reverse the effect of the spores on the monolayers. There was no significant difference between monolayers with isolate 2417 spores with isolate 2417 culture filtrate and the control of isolate 2417 spores alone, but highly significant differences between spores with filtrate together and filtrate alone. Significant differences also occurred between the control (Czapek Dox) and the spores and filtrate together.

This result indicates that although the spores of isolate 2417 promote haemocyte aggregation, the filtrate of this isolate, unlike filtrate from isolate 304, is unable to cause a significant reduction in the nodular response.

Table 30

Comparison of monolayers treated with combined isolate 2417 spores and culture filtrate, with isolate 2417 spores or culture filtrate separately

	10µl of 2417 spores +10µl 2417 culture filtrate	10µl of 2417 spores +10µl CDox	10µl of 2417 culture filtrate + 10µl CDox	20µl CDox
Mean no. of nodules	19.75	29.54	5.875	2.25
SEM	0.2886	2.008	0.5805	0.4392
p v spores	0.223			
p v filtrate	0.000			
p v C-Dox	0.000			

(n = 24)

Haemocyte monolayers were prepared from the haemolymph of 8 first day fifth instar larvae as before. Ten microlitres of conidiospores in GIM from isolate 2417 with a concentration of 1.9×10^5 spores per ml were then added to each monolayer together with 10µl of filtrate from the 8 day growth of isolate 2417 which had been re-filtered through 0.8 / 0.2 µm Acrodisc (Gelman Sciences).

The monolayers were incubated for 4 hours at 22°C ± 1°C and 100% RH and then examined under phase contrast at × 400 with an Olympus BH2 microscope. The number of haemocyte nodules (5 or more contiguous cells in 10 fields was recorded and the work repeated 3 times (n = 24).

Probabilities were calculated with unstacked one-way ANOVA.

The effect of Isolate 304 culture filtrate on nodule formation was so marked that numbers used were high, n = 24, for confirmation. Table 30 shows that this suppressive effect does not occur with filtrate from the culture of Isolate 2417.

4.3.9 Comparison of the effect of fungal culture filtrates and eicosanoids on nodule production by haemocytes *in vitro*

Miller *et al.* (1999) showed that dexamethasone and indomethacin could inhibit the nodule response to infection by the adult cricket, *Gryllus assimilis, in vivo* and that arachidonic acid could reinstate the nodulation response.

In the current work haemocyte monolayers of *M. sexta* were used to compare the effects of fungal filtrate with indomethacin, dexamethasone and arachidonic acid.

a) Dexamethasone and indomethacin compared with culture filtrate from isolate 304

Since nodule formation *in vivo* is promoted by infection, the first series of experiments compared the effect of eicosanoids and fungal filtrate on monolayers treated with 2417 spores. Results are shown in Table 31.

Table 31

Comparison of the effects of indomethacin, dexamethasone and culture filtrate from isolate 304, on nodule production by monolayers

	10µl Indomethacin ($260mg^{-1}$)+10µl 2417 spores	10µl Dexamethasone ($260mg^{-1}$)+10µl 2417 spores	10µl Culture filtrate 304 + 10µl 2417 spores	10µl Czapek Dox + 10µl 2417 spores
Mean no.nodules	8.3	7.2	2.26	21.83
SEM	3.20	2.78	2.26	2.86
p v filtrate and spores	0.048	0.055		
p v spores and C.Dox	0.084	0.031	0.000	

(n = 6)

Monolayers of haemocytes were prepared by chilling first day fifth instar larvae, wiping with 70% ethanol and removing the horn. The haemolymph was collected in microcentrifuge tubes containing 30µl of anti-coagulant at 4°C. It was

centrifuged at *77g* for 5 minutes, the supernatant removed and replaced with GIM. After gentle mixing the haemocyte suspension was placed on sterile cavity slides.

Spores of isolate 2417 were harvested, washed and re-suspended in GIM. Ten microlitres of 3×10^5 spores per ml were added to each monolayer. Culture liquid from the 8 day growth of isolate 304 in C Dox was filtered through sterile muslin and then through 0.8 / 0.2 μm Acrodiscs (Gelman Sciences). Ten microlitres was added to the first control monolayer, whilst 10μl of sterile C.Dox liquid was added to the second control monolayer.

Twentysix milligrammes of dexamethasone and indomethacin (Sigma) were each dissolved in 10ml of 95% ethanol. The solutions were then diluted to 1/10 in GIM and 10μl added to the monolayers with spores.

The monolayers were incubated at 22°C ± 1°C and 100% RH for 4 hours after which the number of haemocyte nodules (aggregates of more than 5 haemocytes) in 10 microscope fields at × 400 were counted.

Each trial contained 2 monolayers for each treatment and was repeated 3 times ($n = 6$).

Probability indicated by analysis of variance (p) showed that dexamethasone had a significant ($p<0.05$) effect on nodule formation, but that the effect of culture filtrate from isolate 304 was greater ($p<0.01$).

b) Dexamethasone and indomethacin compared with culture filtrate from isolate 304 (without spores)

Conidiospores of isolate 2417 had already been shown to promote haemocyte aggregation so a second trial was designed to investigate the effects of dexamethasone, indomethacin and culture filtrate of isolate 304 on haemocyte nodule formation in the absence of spores. Additionally the effects of combinations of filtrate, dexamethasone and indomethacin were studied.

Table 32 shows the difference in nodule formation by haemocytes after treatment. One-way analysis of variance shows that the number of nodules formed after treatment with indomethacin, dexamethasone, or culture filtrate was significantly ($p<0.01$) different from the control.

Table 32

Comparison of effects of indomethacin, dexamethasone and culture filtrate from isolate 304 on haemocyte monolayers without spores

	10µl Indomethacin ($260mg^{-1}$)	10µl Dexamethasone ($260mg^{-1}$)	10µl Culture filtrate 304	10µl Isolate 2417 spores 3×10^5
Mean no.nodules	7.3	2.25	3.0	39
SEM	0.43	0.85	0.70	2.58
p v spores	0.000	0.000	0.000	
p v filtrate	0.391	0.277		

(n = 6)

Monolayers of haemocytes from 2 first day fifth instar larvae were set up for each treatment.

The larvae were chilled at 4°C for 5 minutes, wiped with 70% ethanol and the horn removed. The haemolymph was collected in microcentrifuge tubes containing 30µl of anti-coagulant buffer at 4°C and was centrifuged at 77*g* for 5 minutes. The supernatant was removed and the cells re-suspended in GIM.

Sixty microlitres of cell suspensions were placed on sterile cavity slides and 10µl of indomethacin, dexamethasone, or culture filtrate from isolate 304 added. These solutions were made by dissolving 26mg of dexamethasone or indomethacin in

10 ml of 95% ethanol and diluting this to 1/10 in sterile GIM. The filtrate was from the 8 day growth of isolate 304 in liquid Czapek Dox.

The control monolayer was treated with 10µl of a spore suspension of isolate 2417 at 3×10^5 spores per ml in GIM.

The monolayers were incubated at 22°C ± 1°C and 100% RH for 4 hours. They were then examined under phase contrast with an Olympus microscope at × 400 magnification and the number of nodules (aggregates of more than 5 haemocytes) in 10 fields counted.

The work was repeated three times (n = 6) for each treatment.

c) Effect of combinations of indomethacin, dexamethasone and culture filtrate from isolate 304, on nodule production

Combinations of indomethacin, dexamethasone and culture filtrate of isolate 304 on monolayers treated with spores of isolate 2417 caused the formation of significantly ($p<0.01$) fewer nodules than the individual substances on their own. Results of the combinations of culture filtrate, dexamethasone and indomethacin are compared in Table 33.

Table 33

Comparison of the combined effects of indomethacin, dexamethasone and culture filtrate from isolate 304 on nodule production (with spores)

	10µl Ind + 10µl Filt. + 10µl 2417 spores	10µl Dex. + 10µl Filt. + 10µl 2417 spores	10µl Dex. + 10µl Ind. + 10µl 2417 spores	20µl Filt. + 10µl 2417 spores	10µl Filt. + 10µl 2417 spores	20µl CDox + 10µl 2417 spores
Mean no.of nodules	0.5	0.66	2.66	3.00	2.26	21.83
SEM	0.27	0.36	0.76	0.58	0.51	2.86
p versus 10µl Filt.	0.596	0.519	0.196	0.159		
p versus CDox	0.000	0.000	0.000	0.000	0.000	

(n = 6)

Monolayers of haemocytes from 2 first day fifth instar larvae were set up for each treatment. The larvae were chilled at 4°C for 5 minutes, wiped with 70% ethanol and the horn removed. The haemolymph was collected in microcentrifuge tubes containing 30µl of anti-coagulant buffer at 4°C and then centrifuged at 77*g* for 5 minutes. The supernatant was removed and the cells re-suspended in GIM.

Sixty microlitres of cell suspensions were placed on sterile cavity slides. Ten microlitres of isolate 2417 spores at 3×10^5 spores per ml in GIM were added to each monolayer.

Solutions of dexamethasone and indomethacin were made by dissolving 26mg in 10ml of 95% ethanol and then diluting this 1/10 in sterile GIM. Culture filtrate was from the 8 day growth of isolate 304 in Czapek Dox filtered through sterile muslin and then re-filtered through 0.8 /0.2μm Acrodiscs (Gelman Sciences) to ensure no fungal remnants remained

The monolayers were treated with 20μl combinations of indomethacin, dexamethasone and culture filtrate from isolate 304, with 20μl CDox as a control. They were incubated at 22°C ± 1°C and 100% RH for 4 hours.

The number of haemocyte nodules (more than 5 contiguous haemocytes) in 10 fields was counted with an Olympus BH2 microscope under phase contrast at × 400 magnification,

The work was repeated 3 times (n = 6 for each treatment) and probability calculated with one-way ANOVA.

There was a significant ($p<0.01$) difference between the number of nodules formed in the control (C.Dox) and those formed in the treatment monolayers. Culture filtrate from isolate 304 significantly ($p<0.01$) reduced the number of nodules formed, but the amount of culture filtrate (10μl or 20μl) did not alter the effect. Possibly the threshold for this effect was a much lower dose.

The combination of dexamethasone and indomethacin produced results which were not significantly ($p>0.05$) different from the filtrate on its own. However, combinations of dexamethasone, or indomethacin with culture filtrate significantly ($p<0.01$) reduced nodule numbers.

d) Effect of arachidonic acid on haemocyte monolayers

Miller *et al.* (1994) showed that the eicosanoid precursor arachidonic acid could reverse the effects of dexamethasone on haemocyte nodule formation in *Manduca sexta* fifth instar larvae *in vivo*. He injected 50µg into each insect which had previously been infected with *Serratia marcescens* and injected with dexamethasone or indomethacin. *In vivo* arachidonic acid reversed the nodule-suppressing effects of dexamethasone and indomethacin.

Since 10µl of a 1/10 dilution of both dexamethasone and indomethacin reduced the nodule formation of haemocyte monolayers (Tables 32 and 33), similar concentrations and quantities were used for the *in vitro* work with arachidonic acid.

Table 34

The effect of arachidonic acid on haemocyte monolayers

	10µl arach acid (20mg per ml) + 10µl GIM	10µl arach acid (20mg per ml) + 10µl 2417 spores	10µl 2417 spores + 10µl GIM	10µl C Dox + 10µl GIM
Mean no.of nodules	3.66	23	21.83	3.75
SEM	0.75	2.16	2.86	0.63

(n = 6)

Haemocyte monolayers were prepared by chilling 2 first day fifth instar larvae for each treatment. The insects were wiped with 70% ethanol and the horn removed with flamed scissors.

The haemolymph was collected in microcentrifuge tubes containing 30µl of anti-coagulant buffer at 4°C. The cell suspension was centrifuged at 77*g* for 5 minutes, the supernatant removed and replaced with GIM at 4°C. The haemocytes were gently re-suspended and placed on sterile cavity slides.

100mg of arachidonic acid (Sigma) was dissolved in 500µl of 95% ethanol and then diluted 1 /10 in GIM. Ten microlitres of this dilution was added to the first

monolayer, together with 10µl of GIM, to assess the ability of arachidonic acid to promote nodule formation. The second monolayer had 10µl of arachidonic acid dilution and 10µl of spores of isolate 2417 at 3×10^5 spores per ml in GIM. The third monolayer (1st control) had 10µl of spores of isolate 2417 and 10µl of GIM, whilst the fourth (2nd control) had 10µl of liquid C Dox and 10µl of GIM.

The monolayers were incubated for 4 hours at 22°C ± 1°C and 100% RH. They were then examined with an Olympus BH2 microscope under phase contrast and the number of haemocyte nodules (5 or more contiguous cells) in 10 fields counted.

The work was repeated 3 times for each treatment ($n = 6$).

The probability shown by the one-way analysis of variance of arachidonic acid, rather than spores, causing haemocyte aggregation is $p < 0.001$. The results of treating *M. sexta* monolayers with arachidonic acid alone showed that it did not promote nodule formation *in vitro* (Table 34)

e) Effect of arachidonic acid with dexamethasone or indomethacin, or culture filtrate on haemocyte aggregation

Whilst arachidonic acid on its own did not appear to alter the number of nodules produced by haemocytes, its ability to recover the nodule-forming effect of haemocytes treated with indomethacin, dexamethasone or fungal filtrate was tested.

Table 35

The effect of arachidonic acid with dexamethasone, indomethacin, or fungal filtrate from isolate 304 on haemocyte aggregation

	10µl 2417 spores + 10µl dex.+ 10µl a.acid	10µl 2417 spores + 10µl ind. + 10µl a.acid	10µl 2417 spores + 10µl filt. + 10µl a.acid	10µl 2417 spores + 10µl filt. + 10µl GIM	10µl 2417 spores + 10µl CDox +10µl GIM
Mean no.nodules	3.5	2.25	5.75	3.0	16.5
SEM	0.86	0.47	0.75	1.77	0.64
p	0.000	0.000	0.000	0.000	

(n = 6)

Probability (p) was calculated using ANOVA and shows the liklihood of the difference of the number of nodules in the controls and experimentals occurring by chance. Haemocyte monolayers were prepared by chilling 2 first day fifth instar larvae for each treatment. The insects were wiped with 70% ethanol and the horn removed with flamed scissors.

Haemolymph was collected in microcentrifuge tubes with 30µl of anti-coagulant buffer at 4°C. The cell suspension was centrifuged at 77*g* for 5 minutes, the supernatant removed and replaced with GIM at 4°C. The haemocytes were gently re-suspended and placed on cavity slides.

One hundred milligrammes of arachidonic acid (Sigma) was dissolved in 500µl of 95% ethanol and then diluted 1/10 in GIM. Solutions of dexamethasone and indomethacin were made by dissolving 26mg in 10ml of 95% ethanol and then diluting to 1/10 in sterile GIM.

Culture filtrate from the 8 day growth of isolate 304 was re-filtered through 0.8 / 0.2μm Acrodiscs (Gelman Sciences) to ensure the removal of any fungal fragments.

Spores from isolate 2417 were washed, counted (2×10^5 spores per ml) and suspended in GIM. Ten microlitres of spore suspension was added to 60μl of haemocytes, with 10μl of arachidonic acid and 10μl of either indomethacin or culture filtrate from isolate 304. The control monolayers had either (1)10μl of spore suspension, 10μl of culture filtrate 304 and 10μl of GIM, or (control 2) 10μl spore suspension, 10μl Czapek Dox and 10μl GIM.

The monolayers were incubated for 4 hours at 22°C ± 1°C and 100% RH. The number of haemocyte nodules (aggregations of 5 or more cells) in 10 fields was counted under phase contrast at × 400 magnification.

The work was repeated 3 times (n = 6) for each treatment. One way analysis of variance was used to show the probability of difference between treatments and the Czapek Dox control.

The results given in Table 32 show that dexamethasone and indomethacin depressed the nodule-forming response of haemocytes. The effects of combinations of dexamethasone, indomethacin and culture filtrate on the monolayers (Table 33), were not significantly different ($p>0.05$) from each other, although they were significantly less than the controls ($p< 0.01$).

The addition of arachidonic acid to the haemocyte layers (Table 34) showed that, at these concentrations, *in vivo*, it did not significantly ($p>0.05$) promote nodule formation. The cell aggregation, in this case, appeared to depend on the presence of conidiospores.

Although dexamethasone, indomethacin and culture filtrate 304 all depressed the nodule-forming response, the addition of arachidonic acid to the monolayers (Table 35) did not significantly ($p>0.05$) increase nodule formation.

If arachidonic acid acted *in vitro* in the same way that Miller (1999) recorded *in vivo* one would have expected monolayers treated with eicosanoids or fungal filtrate to have recovered their ability to form nodules.

4.3.10 Effect of culture filtrate 304 dilution on the nodule production by larval haemocytes

Culture filtrate from isolate 304 had a pronounced effect on the formation of nodules by haemocyte monolayers treated with 2417 conidiospores. Dilutions of the filtrate with sterile GIM showed that the causative agent was active up to 1:50 dilution, but not at 1:100 dilution (Table 36), suggesting that its activity was dose-dependent.

Controls with Czapek Dox and GIM replacing culture filtrate 304 showed that there was no significant difference of effect on nodule formation between the two media ($p = 0.26$). Equally, suspending the spores in C.Dox or GIM had no significant effect ($p = 0.51$) on nodule formation and it seems unlikely that the different culture media influenced the effect of the filtrate.

Table 36

Number of nodules produced by monolayers of haemocytes treated with isolate 2417 spores and different concentrations of isolate 304 culture filtrate

Each treatment included 10µl 2417 spores at 2×10^5 ↓	Mean No of Nodules per 10 fields	SEM
10µl Whole culture filtrate	3.9	0.4914
10µl 1/5 dilution culture filtrate	4.75	0.6364
10µl 1/10 dilution culture filtrate	8.56	1.1314
10µl 1/25 dilution culture filtrate	14.56	1.8385
10µl 1/50 dilution culture filtrate	14.0	1.7677
10µl 1/100 dilution culture filtrate	22.25	2.4006
Control (1) 10µl 2417 spores. Filtrate replaced with 10µl CDox	22.31	1.4673
Control (2)10µl 2417 spores Filtrate replaced with10µl GIM	20.8	1.4672
Control (3) spores and filtrate replaced with 20µl C Dox	2.8	0.6116
Control (4) spores and filtrate replaced with 20µl GIM	1.68	0.5727

(n = 8)

For each treatment two first day fifth instar larvae were chilled, wiped with 70% ethanol and the horn removed. The haemolymph was collected in microcentrifuge tubes with 30µl of anti-coagulant buffer at 4°C and centrifuged at 77*g* for 5 minutes. The supernatant was removed and replaced with GIM and monolayers set up on cavity slides.

Spores of isolate 2417 were harvested, washed, re-suspended in GIM and counted (2×10^5 spores per ml). Ten microlitres of spore suspension was added to each monolayer.

Culture filtrate from the 8 day growth of isolate 304 in liquid Czapek Dox was filtered through sterile muslin and then through 0.8/0.2µm Acrodiscs (Gelman

Sciences). Dilutions of this filtrate were made in sterile GIM and 10µl added to the monolayers.

Control monolayers were set up (1) with 2417 spores and CDox replacing culture filtrate; (2) with 2417 spores and GIM replacing culture filtrate; (3) spores and filtrate replaced with CDox and (4) spores and filtrate replaced with GIM.

All the monolayers were incubated for 4 hours at 22°C ± 1°C and 100% RH. They were examined at × 400 under phase contrast with an Olympus BH2 microscope and the number of nodules (aggregates of 5 or more cells) in 10 fields counted.

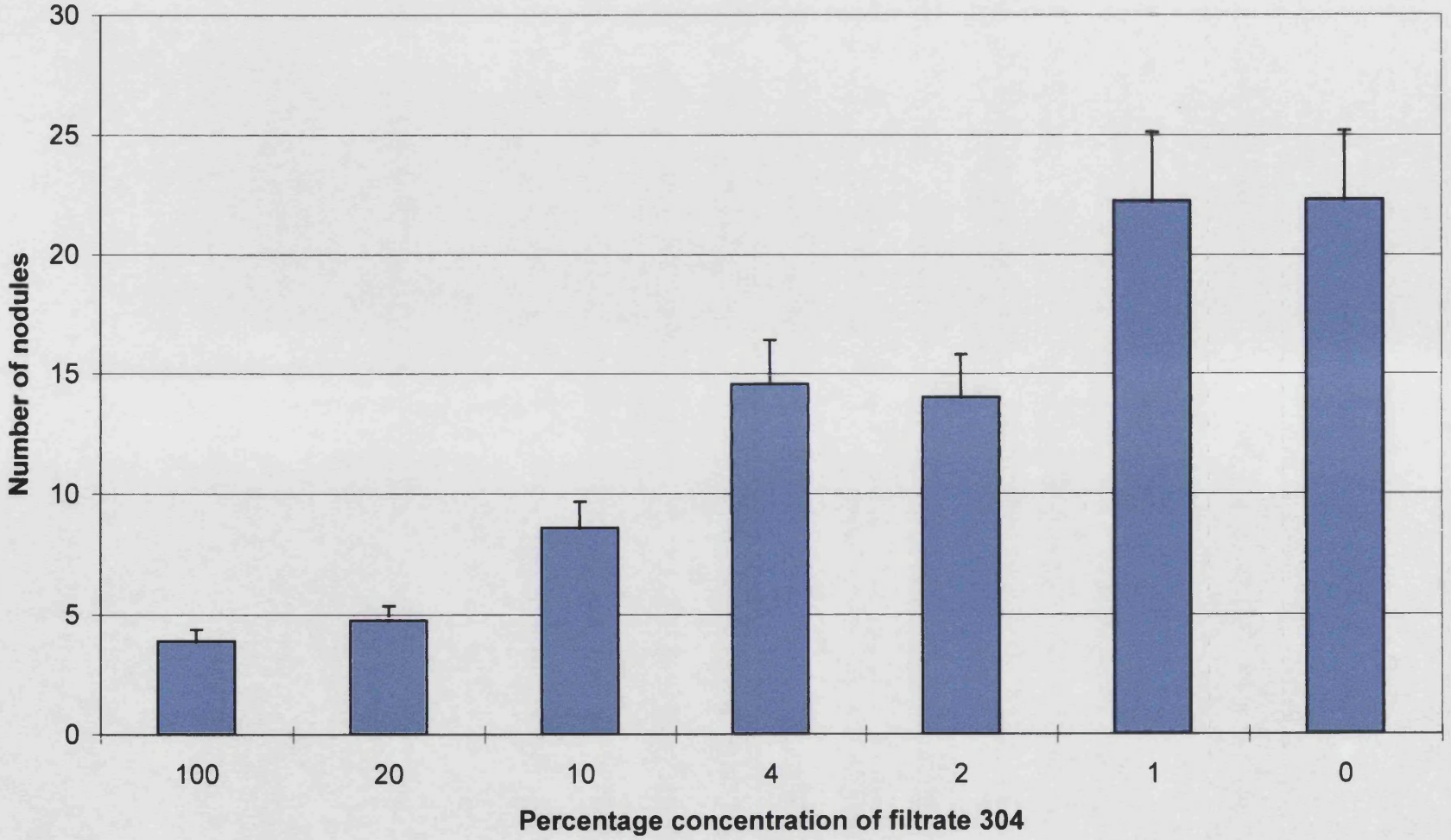
The work was repeated 4 times for each treatment (n = 8).

Figure 18 (overleaf) suggests that even at 100% concentration, culture filtrate from isolate 304 was unable to totally inhibit nodule production, but that at a 1:50 dilution the filtrate was still effective. Although nodule production after monolayer treatment with spores is clearly dependent on filtrate dose, a low level of nodules produced in untreated monolayers may indicate the involvement of more than one factor in nodule formation.

Fig. 18

This figure shows the number of haemocytic nodules (aggregations of 5 or more cells) formed by monolayers treated with 10µl of 2417 spores (2×10^5 per ml) and 10µl of different dilutions of culture filtrate 304. In the control (0) the culture filtrate was replaced with 10µl of Czapek Dox. Monolayers were incubated at 22°C for 4 hours and the number of nodules in 10 microscope fields at × 400 counted.

Fig. 18. Effect of filtrate 304 concentration on nodule formation



n = 8

4.3.11 Effects on nodule formation of different fractions of culture filtrate from isolate 304 after ultra-filtration

In order to determine the size of the metabolites which prevented the formation of haemocytic nodules the filtrate was passed through a membrane filter device (Ultrafree Centrifugal Device, Millipore, Bedford MA) with a cut-off pore size of 10kDa. Initially the different fractions of culture filtrate from isolate 304 gave results which were unclear. However, by washing the retained fraction (more than 10kDa) and diluting both the larger and smaller fractions (more and less than 10kDa) it was possible to show that the active agent preventing haemocyte aggregation was smaller than 10 kDa .

Table 37 shows the number of nodules produced by haemocyte monolayers after treatment with dilutions of culture filtrate of isolate 304 which had been passed through a 10kDa membrane.

For each treatment, four first day fifth instar larvae were chilled, wiped with 70% ethanol and the horn removed. The haemolymph was collected in microcentrifuge tubes containing 30µl of anti-coagulant buffer at 4°C. The tubes were centrifuged at 77*g* for 5 minutes, the supernatant removed and replaced with GIM at 4°C and the cells re-suspended and placed on cavity slides .

Spores of isolate 2417 were harvested, washed, suspended in GIM and counted (2×10^5 spores per ml). Ten microlitres of spore suspension was added to each monolayer.

Culture filtrate from the 8 day growth of isolate 304 was filtered through sterile muslin and then placed in a membrane device (Millipore,Bedford MA) with a 10kDa cut-off point. The culture filtrate was centrifuged at 1407*g* for 20 minutes and dilutions were made in Czapek Dox. Ten microlitres of the appropriate dilution was added to the monolayers which were incubated at 22°C ± 1°C for 4 hours. The number of haemocyte nodules (aggregations of 5 or more cells) in 10 microscope fields at × 400 magnification were counted.

The work was repeated 3 times for each treatment (n = 12).

Table 37

Number of nodules produced by haemocytic monolayers treated with isolate 2417 spores and dilutions of two fractions of culture filtrate 304

	Less than 10 kDa			More than 10 kDa	
	Mean no. of nodules	SEM	ANOVA p	Mean no.of nodules	SEM
10µl undiluted filtrate fraction + 10µl 2417 spores	1.667	0.497	0.003	7.5	1.7078
10µl1/5 dilution culture filtrate + 10µl 2417 spores	2.25	0.6976	0.000	14.25	2.2998
10µl 1/10 dilution culture filtrate + 10µl 2417 spores	4.33	0.6665	0.000	16.583	1.5247
10µl ½5 dilution culture filtrate + 10µl 2417 spores	4.835	0.894	0.000	15.66	1.8062
10µl1/50 dilution culture filtrate + 10µl 2417 spores	9.916	1.083	0.000	16.25	0.4817
10µl1/100 dilution culture filtrate + 10µl 2417 spores	15.083	0.7432	0.784	15.5	1.3055
10µl C.Dox + 10µl2417 spores	22.3	1.054		22.3	1.055
10µl whole filtrate (both fractions together) + 10µl 2417 spores	1.9	0.4991		1.9	0.4991

(n = 12 for each treatment)

Effect of dilution on the two fractions of the filtrate

An unstacked one-way analysis of variance (Minitab) between the two filtrate fractions showed that their effects on the haemocytes were significantly different from each other ($p < 0.001$) up to 1:100 dilution. At this point there was no significant difference ($p = 0.784$) between the two fractions and it seemed probable that the active metabolite was too dilute to be effective in either fraction.

One-way analysis of variance showed that at 1:5 dilution the < 10kDa fraction was not significantly different ($p = 0.503$) from the undiluted filtrate. As dilution increased the number of nodules on the monolayer rose and at 1:50 filtrate dilution was significantly different from the undiluted filtrate, but not significantly different ($p>0.05$) from the control of Czapek-Dox.

With the diluted filtrate fraction of "more than 10kDa" there was no significant difference ($p>0.05$) between any of the dilutions and the Czapek-Dox control. However, there was a significant difference ($p<0.05$) between the number of nodules formed after treatment with the undiluted fraction and the control. This may have been due to some of the aggregation-suppressing factor, from the "smaller than 10kDa" fraction, being trapped with the larger molecules in the upper part of the filter.

The results in Table 37 clearly show that a nodule-inhibiting factor of less than 10kDa exists in the culture filtrate of isolate 304 and that the inhibition of nodule formation is dose dependent on this factor.

4.3.12 Effect of heating culture filtrate from isolate 304 on the aggregation of spore- treated haemocytes

Table 38 shows that heating the < 10kDa fraction of culture filtrate from isolate 304 did not effect its ability to prevent haemocytes in spore-treated monolayers aggregating.

Table 38

Effect of heating culture filtrate from isolate 304 on the nodule production of monolayers treated with isolate 2417 spores

	10µl heated (100°C/20mins) culture filtrate 304+10µl2417 spores	10µl un-heated culture filtrate 304+10µl 2417 spores	10µl Czapek Dox (heated) + 10µl 2417 spores
Mean no.of nodules	3.104	2.416	16.5
SEM	0.2565	0.2774	0.708
p versus control	0.000	0.000	
p versus un-heated filtrate	0.224		

(n = 18 for each treatment)

The one-way analysis of variance (ANOVA) showed that there was no significant difference (p = 0.224) in the number of nodules produced by monolayers treated with heated, or unheated, filtrate (Table 38). One must conclude that heating to 100°C does not affect the active component of the filtrate.

To test the effect of heating the culture filtrate on haemocyte nodulation monolayers were set up as follows. Six first day fifth instar larvae were chilled, wiped with 70% ethanol and the horn removed. The haemolymph was collected in microcentrifuge tubes with 30µl of anti-coagulant at 4°C and then centrifuged at 77*g* for 5 minutes. The supernatant was removed and replaced with GIM at 4°C and the cells placed on sterile cavity slides. Ten microlitres of isolate 2417 spores in GIM (2 × 10^5 spores per ml) were added to the monolayers.

Five ml of culture filtrate from the 8 day growth of isolate 304 was filtered through sterile muslin and divided into two equal aliquots in glass universals. One aliquot was heated to 100°C for 20 minutes in a water bath and cooled to 22°C.

Ten microlitres of the heated filtrate was added to the experimental monolayers and 10µl of unheated filtrate to the controls. A control of 10µl of Czapek Dox, previously heated to 120°C for 20 minutes and cooled to 22°C, was included.

The monolayers were incubated for 4 hours at 22°C ± 1°C and 100% RH. The number of nodules (aggregates of 5 or more cells) in 10 fields at × 400 magnification, were counted.

The work was repeated 3 times (n = 18 for each treatment).

4.3.13 Effect of protease on culture filtrate from isolate 304

In order to further characterise the fungal metabolites in the culture filtrate which prevented haemocytic aggregation the filtrate was treated with a solution of protease P5147 (Sigma). This wide-acting enzyme destroys most proteins likely to be present in fungal culture filtrates and the results of its action are shown in Table 39.

Table 39

Effect of protease treatment on culture filtrate of isolate 304 on the nodule formation of larval haemocytes treated with conidiospores

	10µl protease-treated culture filtrate 304 +10µl 2417 spores	10µl untreated culture filtrate 304+10µl 2417 spores	10µl CDox +10µl 2417 spores
Mean no.nodules	21.1389	3.75	18.722
SEM	1.217	0.3070	0.9002
p versus CDox	0.119	0.000	
p versus untreated culture filtrate	0.000		

(for each treatment n = 18) Probability calculated with one-way ANOVA.

Monolayers of larval haemolymph were prepared by chilling and then wiping first day fifth instar larvae with 70% ethanol. The horn was removed and the haemolymph collected in microcentrifuge tubes containing 30µl of anti-coagulant buffer. These were centrifuged at 77*g* for 5 minutes, the supernatant removed and replaced with GIM at 4°C. 60µl of haemocyte suspension was placed on sterile cavity slides.

Spores of isolate 2417 were harvested, washed, counted (2.2×10^5 spores per ml) and re-suspended in GIM. Ten microlitres of spores was added to each monolayer. Culture filtrate from the 8 day growth of isolate 304 in liquid Czapek Dox was filtered through sterile muslin and placed in a membrane filter device (Millipore, Bedford MA) with a 10kDa pore size. The filtrate was centrifuged at 1407*g* for 20 minutes and the fraction that passed through the membrane (<10kDa) was collected.

The wide-acting protease (P5147 Sigma) was made up by dissolving 100mg in 100ml of PBS at pH 7.2. One hundred millilitres of the prepared culture filtrate

was mixed with 100ml of protease solution and incubated at 37°C for 30 minutes. The "smaller-than-10kDa" fraction was re-passed through a 10kDa membrane assembly after treatment with the wide-activity protease (molecular weight 22kDa) and centrifuged for 20 minutes at 1407*g* to remove any protease from the filtrate and avoid any effects of the enzyme itself on haemocytic membranes. Finally the treated fraction was heated to 100°C for 20 minutes to de-activate any remaining protease.

Ten microlitres of the treated filtrate was added to the prepared monolayers, with controls of (1) unheated filtrate and (2) liquid Czapek Dox. The monolayers were incubated at 22°C ± 1°C and 100% RH for 4 hours and then examined at × 400 magnification. The number of haemocyte nodules (aggregates of 5 or more cells) in 10 fields was counted and the work repeated 3 times (n = 18 for each treatment).

Protease treatment had a profound effect on the activity of culture filtrate of isolate 304.

Table 39 shows that there was a highly significant difference between the protease-treated filtrate and the untreated filtrate suggesting that the nodule-suppressing element had been de-activated. This was reinforced by the lack of significant difference between the Czapek Dox control and the protease-treated filtrate. The untreated filtrate suppressed nodule production, whilst the protease-treated filtrate and the Czapek Dox control did not affect it.

The clear result was that this wide-acting protease destroyed the ability of the filtrate to suppress nodule production and suggests that the active agent is a small protein, or peptide.

The controls for the protease-treated filtrate included a monolayer with C.Dox and one with untreated filtrate. An extra control monolayer with protease-treated filtrate which had not been passed through a 10kDa membrane was also included. Whilst the haemocytes of the first three sets of monolayers appeared normal, controls with the protease/filtrate solution which had not passed through the 10kDa membrane were distinctly different. The total haemocyte count was halved and cells were misshapen with exaggerated spindle-shapes. There was also evidence of considerable cell debris. Spores (added to promote nodulation) appeared unchanged by the protease treatment.

4.3.14 Use of the Coomassie Blue protein assay (Bradford Test) to compare the amounts of protein in treated culture filtrates from isolates 304 and 2417

In the use of this assay, the relationship between the absorbance of the dye and protein concentration is non-linear, but by plotting the log of the absorbance against the log of the protein concentration in the standard, a straight line can be obtained. The assay is sensitive to the presence of 16μg of protein or more, per ml. and was used to compare the amounts of protein in the culture filtrates of isolates 304 and 2417. The results are shown in Table 40.

Table 40

Comparison of the amount of protein in heated culture filtrates of <10kDa from isolates 304 and 2417

Culture filtrate from isolate 304		Culture filtrate from isolate 2417	
Dilution	μg protein /ml	Dilution	μg protein/ml
No dilution	51.2	No dilution	22.5
1/5 dilution	< 16	1/5 dilution	<16
1/10 dilution	< 16	1/10 dilution	<16

The results are the mean values of 3 replicate assays. No readings were available with either isolate at 1/5 dilution or below since the Bradford test is only sensitive to 16μg of protein per ml and the amount of protein present in these dilutions was less than this.

Filtrates from the 8 day growth of isolates 304 and 2417 in liquid Czapek Dox were assayed for protein content. The cultures were first filtered through sterile muslin and then through 0.8/0.2μm Acrodiscs (Gelman Sciences). Five ml of culture filtrate from each isolate was placed in a membrane filter device with a 10kDa pore size (Millipore, Bedford MA) and centrifuged at 1407*g* for 20 minutes. The <10kDa fractions of both isolate culture filtrates were then heated to 100°C for 20 minutes in a water bath and cooled to 22°C. Dilutions of these filtrates were made in distilled water (1/5 ;1/10 ;1/25 and 1/50).

Twenty microlitres of each dilution was placed in a microcentrifuge tube and 50μl of 1M NaOH and 1ml of Coomassie Brilliant Blue dye solution added. The tubes were then inverted to mix the contents and incubated at 22°C for 5 minutes.

Two hundred microlitre samples of each dilution were added to the wells of a microtitre plate, with distilled water as a blank. The absorbance was read at 595nm with a Dynatech MR 500 plate reader.

The results were compared with a standard curve made from dilutions of Bovine Serum Albumen and the amount of protein in the culture filtrate samples calculated.

Certain proteins are known to produce comparatively low absorbance levels (Stoscheck 1990) and it is possible that the proteins in the fungal filtrates were similar. Since the culture filtrates from the growth of *B. bassiana* contained an un-characterised protein which had to be compared with the standard curve for bovine serum albumen, the results are arbitary. However, the Bradford assay showed that there was a considerable difference in the amount of heat-stable protein of less than 10kDa in culture filtrates of isolates 304 and 2417.

These results led to the use of gel electrophoresis (SDS-PAGE) to determine whether the proteins present were different in the two culture filtrates, or merely in different concentrations.

4.3.15 Gel electrophoresis (SDS PAGE) of culture filtrates from isolates 304 and 2417

The Bradford assay had shown that small, heat-stable proteins were present in the culture filtrates of isolate 304 and 2417 so SDS Page gel electrophoresis was used to further define them.

Samples of culture filtrate from the 8 day growth of isolates 304 and 2417 were passed through a membrane filter device (Millipore, Bedford MA) with a 10kDa pore size by centrifuging at 1407*g* for 20 minutes. The < 10kDa filtrates of both isolates were heated to 100°C for 20 minutes, cooled and 2ml aliquots freeze-dried (Savant Speed Vac concentrator with Edwards Freeze Dryer Modulyo vacuum pump). The dried residues were re-suspended in distilled water to give samples of 30, 20, 15, and 10 times the original concentration after the addition of loading buffer. The samples and the broad range marker (BioRad 6.1kDa-200kDa, 9 different proteins) were heat-shocked at 100°C for 3 minutes. Twenty microlitre samples were loaded onto an SDS PAGE gel which was run for approx. 1 hour.

The completed gels were fixed and stained for 16 hours with Coomassie Brilliant Blue (Sigma R250) in 1:1 methanol with 10% glacial acetic acid. They were then de-stained with 3 changes of 1:1 methanol with 10% glacial acetic acid.

Silver staining was used to visualise the lower concentrations of protein since this method is more sensitive than staining with Coomassie Brilliant Blue. Gels were fixed for 16 hours with a solution of 50% methanol, 12% acetic acid and 0.5ml per litre of 37% formaldehyde and then washed 3 times with 50% ethanol. They were soaked for 1 minute in 0.02% sodium thiosulphate to sensitise them and then rinsed 3 times with distilled water. The gels were then stained in a solution of 10% silver nitrate with 150µl of 37% formaldehyde in 200ml of distilled water for 20 minutes.

When the protein bands became visible the development was halted with a 50% solution of methanol in distilled water with 12% acetic acid added.

The results of staining the protein bands are shown in Plates 11 and 12.

Coomassie Brilliant Blue stained a protein band in proportion to the concentration of culture filtrate from isolate 304. Compared with the wide-range marker the protein was estimated to be slightly smaller than 6,500 Daltons, the size of the smallest protein in the marker, aprotinin. No protein bands were stained with Coomassie Blue with culture filtrate from isolate 2417.

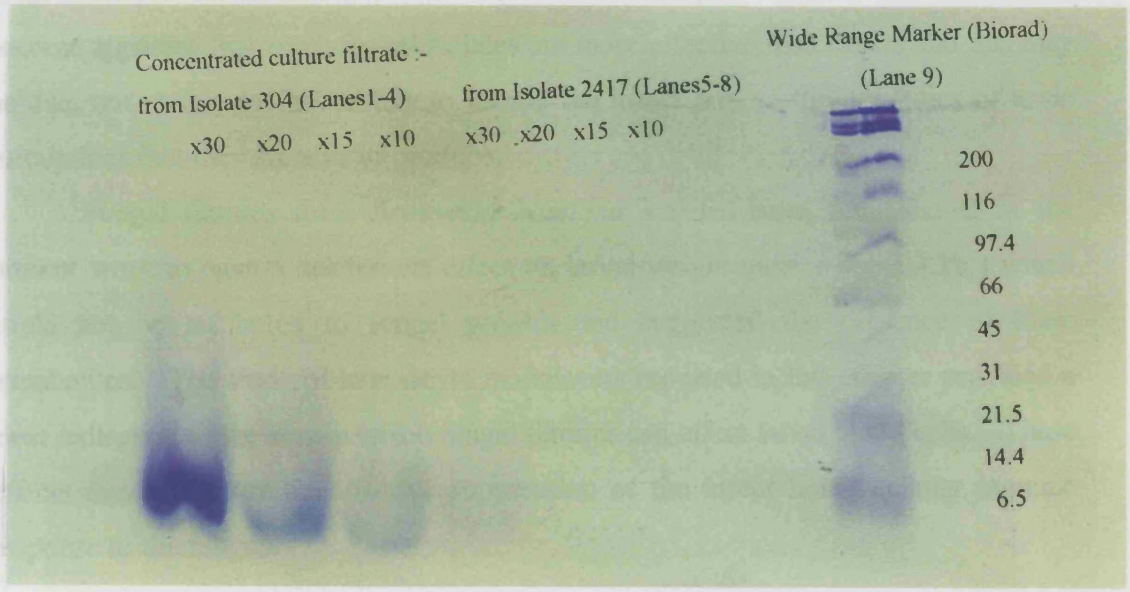
Silver nitrate staining is reputedly 100 times more sensitive than Coomassie blue and is said to be able to detect as little as 0.38 ng/ml of bovine serum albumen (Hames and Rickwood, 1990). However, not all proteins stain well with silver stain and it is difficult with uncharacterised proteins to be sure that they have all been detected. The silver stain was used to determine if there were small amounts of protein in the culture filtrate of isolate 2417 which had not stained with Coomassie Brilliant Blue.

Bands of decreasing intensity of a small protein were apparent after silver staining dilutions of culture filtrate from isolate 304 (Plate 12). Additionally faint bands of larger proteins were evident and suggest that small amounts had been able to pass through the 10kDa membrane. Since the samples had been concentrated 30 times the amount of these larger proteins must have been very small. No bands were visible from culture filtrate 2417.

Concentrated culture filtrate :-								Wide Range Marker (Biorad)
from Isolate 304 (Lanes1-4)				from Isolate 2417 (Lanes5-8)				(Lane 9)
x30	x20	x15	x10	x30	x20	x15	x10	
								200
								116
								97.4
								66
								45
								31
								21.5
								14.4
								6.5

Concentrated culture filtrate :-								Wide Range Marker (Biorad)
from Isolate 304 (Lanes 1-4)				Isolate 2417 (Lanes 5-8)				(Lanes 9-10)
x30	x20	x15	x10	x30	x20	x15	x10	
								200
								116
								97.4
								66
								45
								31
								21.5
								14.4
								6.5

Plate 11 Protein bands from culture filtrates of isolates 304 and 1417 stained with Coomassie Blue



Differences in the presence of small proteins were apparent in bands stained with Coomassie Blue or silver nitrate after gel electrophoresis. Filtrate from isolate 2417, the isolate which had no anti-aggregation effect on haemocytes, had no evident protein bands, whilst filtrate from isolate 304 showed a band with concentration-dependent staining.

Plate 12 Silver stained bands of protein from culture filtrates of isolates 304 and 2417

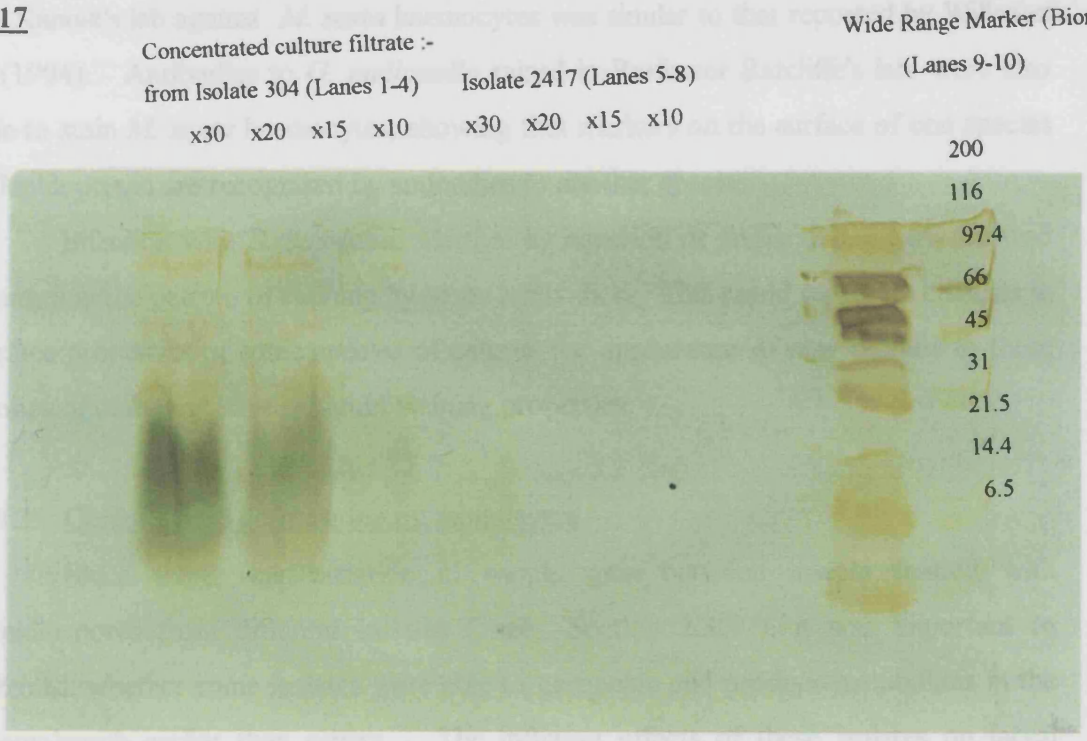
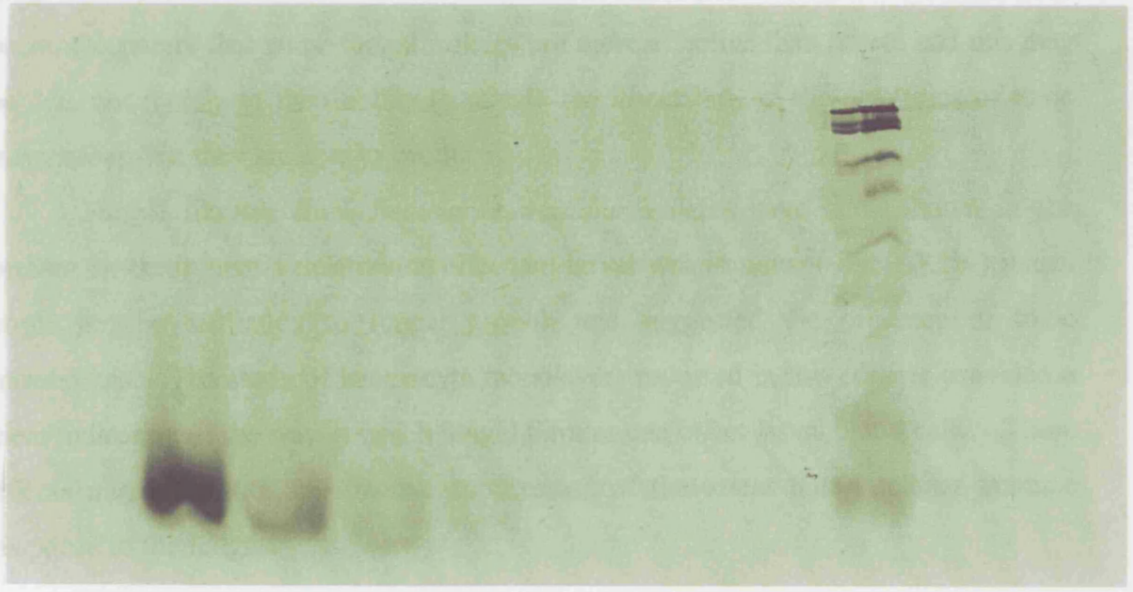


Plate 11 Protein bands from culture filtrates of isolates 304 and 1417 stained with Coomassie Blue



Differences in the presence of small proteins were apparent in bands stained with Coomassie Blue or silver nitrate after gel electrophoresis. Filtrate from isolate 2417, the isolate which had no anti-aggregation effect on haemocytes, had no evident protein bands, whilst filtrate from isolate 304 showed a band with concentration-dependent staining.

Plate 12 Silver stained bands of protein from culture filtrates of isolates 304 and 2417

4.4.1 Discussion

Fungal infection of insects has aroused interest world-wide since pathogenic fungi could provide an alternative to chemicals in insect pest control. However it has become apparent that some fungal isolates are more effective than others and this may be due, not merely to their ability to invade the insect but, to the quantities of toxic metabolites that they are able to produce.

Fungal filtrates from *Beauveria bassiana* isolates have been shown in the present work to have a deleterious effect on larval weight gain (see 3.3.2b) which could not be attributed to fungal growth and suggested the presence of toxic metabolites. The study of haemocyte monolayers reported in this chapter provided a clear indication of the way in which fungal filtrates can effect larval blood cells. These effects may constitute an effective suppression of the insect host's cellular immune response to the fungus.

4.4.2 Antibody staining

Antibody staining was used primarily to distinguish cell types, but differences between the ability of antibodies to stain cells suggested that variations might occur between the cell surfaces. The pattern of staining specificity of the antibodies raised in Dr. Kanost's lab against *M. sexta* haemocytes was similar to that reported by Willot *et al* (1994). Antibodies to *G. mellonella* raised in Professor Ratcliffe's lab. were also able to stain *M. sexta* haemocytes, showing that markers on the surface of one species of lepidopteran are recognised by antibodies to another species.

Infection with *B. bassiana*, whether by injection or immersion, clearly showed changes in the pattern of staining by some antibodies. This could represent changes in surface properties of some species of cells as the appearance of new recruits to these groups of cells may have different staining properties.

4.4.3 Conidiospore germination on monolayers

Since there was variation in weight gain between insects treated with conidiospores from different isolates (see Section 2.3.3), it was important to establish whether some isolates were able to germinate and produce metabolites in the haemolymph earlier than others. The different effects of these isolates on larval

weight gain might be due solely to the inability of their spores to germinate in the larval haemolymph.

Although there was variation in the rate at which conidiospores from different isolates germinated, by 48 hours all the isolates had achieved at least 17% germination and after this point in time it became difficult to pursue the study since the monolayers had become overcrowded by the production of mycelia.

Speed of germination may not be linked with the subsequent rate of mycelial growth and metabolite production. However, the ability of the conidiospores to germinate in larval haemolymph *in vitro* provides a suitable method for the study of fungal/haemocyte interactions. Additionally, a possible complication of anti-fungal metabolites produced by the larval fat body is avoided. *Drosophila* is known to produce a range of anti-microbial peptides in response to infection, but as yet their effect on the germination of fungal spores has not been investigated.

Ability to germinate on haemolymph monolayers was not a factor in the difference between isolates to affect larvae since all the isolates could germinate on the monolayers.

4.4.4 The effect of fungal filtrate on phagocytosis

Hung and Boucias (1993) showed that an isolate of *Beauveria bassiana* (UF1 5477, originally isolated from adult mole crickets) was able to suppress the spreading and phagocytic effects of *Spodoptera exigua* larval haemocytes *in vivo.* It was suggested that the fungus might produce immuno-suppressive substances.

In the present work, phagocytosis studies of *B. bassiana* spores on monolayers of *M . sexta* haemocytes showed that even when an excess of spores was available only 1% of haemocytes undertook phagocytosis. The pre-treatment of fungal spores with rhodamine allowed their phagocytosis to be followed and it was evident that the majority of spores remained in suspension and only a small percentage were engulfed. Even so, the addition of fungal filtrate from the *in vitro* growth of isolate 304 had a significant effect ($p < 0.002$) on phagocytosis, reducing the number of haemocytes undertaking this to less than half.

There seems no doubt that this fungal filtrate contained products which were able to suppress the phagocytic response of haemocytes. Thus the results *in vitro* confirmed the earlier work carried out by Hung *et al.* (1993) *in vivo.*

4.4.5 Haemocyte response to conidia of different *B. bassiana* isolates

It was clear from earlier work *in vivo* (2.3.1 and 2.3.3) that the twelve different *B. bassiana* isolates used had varying effects on the tested insect larvae. Some isolates caused a shorter LT_{50} , or were able to reduce the larval weight gain to a larger extent than others.

The study of these isolates, both *in vivo* and on haemocyte layers *in vitro* has provided additional information about their actions on the insect.

Typically, larval haemocytes began to aggregate one or two hours after injection of spores into the larvae. These results were mirrored *in vitro* when inoculated monolayers were used. This method had the advantage that the development of nodules on the monolayers could be studied over a period of hours and that no secretory products from the larvae interfered.

The nodule response to the spores varied considerably between isolates. Spores from isolate 2417 caused the production of three times more nodules than the average, whilst those from isolate 304 consistently caused the production of fewer nodules than any other, both *in vivo* and *in vitro*. The number of nodules produced in response to this isolate was actually significantly less than the control, and thus strongly suggested the presence of an immuno-suppressive factor.

4.4.6 Conidial concentration and nodule formation

Ratcliffe and Walters (1983) showed that the number of nodules and the speed at which they were formed depended on the dose and pathogenicity of the strain of *Bacillus cereus* injected into *Galleria mellonella* larvae. They suggested that nodule formation was dose-dependent and varied with the pathogenicity of the microorganisms injected.

By contrast, the current work showed that higher concentrations of spores of isolate 304 led to the formation of fewer nodules. As this was one of the most pathogenic isolates its success may have been due to the ability of the spores to reduce, or inhibit, the cellular response to infection.

Isolate 2417 gave quite different results. Here haemocytes responded to increased spore concentrations by producing more nodules. This isolate, which is pathogenic, but has a long LT_{50} and the highest LC_{50} of the isolates tested, does not

appear to owe its limited success as a pathogen to the suppression of the host's cellular immune response.

4.4.7 Effect of heating conidiospores on nodule formation

The effect on nodule formation of heating spores of two different isolates was compared.

When conidiospores of isolate 2417 were heated their previous ability to stimulate nodule production was destroyed. Since the effect was the same both *in vivo* and *in vitro* it was clear that the haemocyte reaction of the larvae was only to the live spores of this isolate.

By contrast, the effect of isolate 304 spores was not significantly altered by heating and there was little difference between the results *in vivo* and *in vitro.* Spores of isolate 304 do not stimulate nodule formation either *in vivo* or *in vitro* and this is also true of heated spores. This could reflect the absence from the surface of the spores of a heat-labile nodule inducing substance that is present on the surface of spores of isolate 2417. Alternatively, the 304 spores may possess a spore-surface nodulation inhibitor. If this were the case it might, or might not, be heat-stable since the factor on the spore surface that induces nodulation is evidently heat labile. If a substance existed, either on the surface of the spores, or exuded during the four hours incubation (and before any visible germination) it was not destroyed by heating. The heat stability or otherwise of a nodular inhibitor would have been revealed by this experiment.

4.4.8 Effects of fungal filtrates on the nodule-forming response of haemocytes

The results of injecting larvae with 2417 filtrate, but without spores, showed that the filtrate alone, unlike the spores, did not stimulate nodule production *in vivo.* Similar results occurred *in vitro* when monolayers were treated with 2417 filtrate alone. Those treated with 2417 spores produced large numbers of nodules, but those treated with filtrate did not.

The most striking results occurred when filtrate from the different isolates was added to monolayers treated with 2417 spores. All but one of the filtrates (2417 itself) exhibited a suppressive effect on the ability of the monolayers to form nodules.

It was apparent that most of the filtrates were able to reduce the aggregation ability of the haemocytes. Culture filtrate from isolate 304, one of the isolates producing the most marked effects, was chosen for further study.

4.4.9 Comparison of effects of eicosanoids or fungal filtrate on haemocyte nodule production

Miller *et al.* (1999) showed that the eicosanoids dexamethasone and indomethacin were able to reduce the nodule response of *Gryllus assimilis* haemocytes *in vivo*. He suggested that the nodule-forming response could be severely impaired by treating crickets with eicosanoid inhibitors prior to infection. The nodule response could be recovered by treatment with the polyunsaturated fatty acid, arachidonic acid. Although most of the earlier work on eicosanoids had been done *in vivo*, Hampson, Rowley, Barrow and Steadman (1992) used blood cells of the crab *Carcinus maenas* to show that eicosanoids and prostaglandins could be produced *in vitro*.

With these results in mind, a series of experiments was designed to ascertain whether, *in vitro, B. bassiana* abrogates the host nodulation response by interfering with eicosanoid metabolism and if the response could be rescued with arachidonic acid.

Results of the present work showed that the eicosanoids indomethacin and dexamethasone reduced nodule formation by *M. sexta* haemocytes. This is in accordance with the previous work of Mandato *et al.* (1997) where dexamethasone and indomethacin were shown to significantly inhibit the nodule formation *in vivo* in *Galleria mellonella* larvae but arachidonic acid was able to reverse the effect and stimulate nodule production. However, *M. sexta* haemocytes *in vitro* did not produce more nodules than the untreated control when arachidonic acid was added. There was little difference in nodule formation between monolayers treated with 2417 spores, those treated with arachidonic acid, and monolayers treated with arachidonic acid and 2417 spores together. There was certainly no evidence to suggest that arachidonic acid promoted nodule formation *in vitro*.

Hampson *et al.* (1992) showed that the blood cells of the crab, *Carcinus maenas,* produced eicosanoids which might effect nodule production. In the current work, the insect *M. sexta* produced nodules as a reaction to spores both *in vivo* and *in vitro,* but the addition of arachidonic acid did not increase nodule production.

4.4.10 Anti-aggregation component of filtrates

All except one of the fungal filtrates tested on the haemolymph monolayers suppressed nodule production by the haemocytes. Culture filtrate of isolate 304 was shown to have one of the most pronounced effects and was used to further characterise the active component which prevented haemocyte aggregation.

Dilutions of one-in-fifty showed that this component was active on the blood cells at considerable dilution. Although there might be only a very small amount of the active substance present the extreme reaction of the haemocytes gave a clear indication of its presence and could be used to test for activity after different filtrate treatments.

The use of membrane filters allowed the separation of the fungal filtrate into fractions smaller, or larger, than 10kDa. After dilution it was clear that almost all the aggregation factor lay in the less than 10kDa fraction. This method of separation allowed a slight amount of retention of the smaller molecule with the larger fraction, but its effect on the haemocyte aggregation disappeared on dilution (see page 190).

Heating the "smaller-than 10 kDa fraction" of filtrate had no effect on its ability to prevent nodulation of haemocytes. By contrast, treatment with a wide-acting protease (which was subsequently removed) completely destroyed this ability.

The Bradford assay showed a considerable difference in the amount of protein in culture filtrates from isolates 304 and 2417. The difference in their effect on the aggregation of larval haemocytes might be entirely due to the concentration of a particular protein. Alternatively it might be due to the presence of different proteins in these two isolates. SDS PAGE gel electrophoresis showed that the filtrate from isolate 304 contained appreciable quantities of a small protein which was not apparent in the filtrate from isolate 2417. This protein from the filtrate of isolate 304 could be concentrated and recovered with a native gel and might then be used to assay to assay for haemocyte aggregation.

Thus the factor present in the filtrate which was able to suppress haemocyte nodulation appeared to be a small, heat-stable protein produced in varying amounts by the different isolates of *Beauveria bassiana.*

Chapter 5

Discussion

5.1 A complex host-pathogen relationship

The effects of the spores of entomopathogenic fungi on their insect hosts are subtle and diverse. The reactions of *Manduca sexta* larvae to a range of lepidopteran isolates of *Beauveria bassiana* typify the complexity of the host-parasite relationship.

Work undertaken early in the course of this research showed that conidiospores of some isolates of *B. bassiana* were able to germinate, form appressoria, penetrate the insect cuticle then, by using the available nutrients in the haemolymph, develop rapidly, killing the larvae by mycosis before pupation. These isolates followed the pattern of effective entomopathogens. By contrast, spores of other isolates which were investigated, although unable to penetrate the insect cuticle, were effective once injected into the haemocoel. They were able to cause loss of weight gain at low doses and insect death by mycosis at high doses.

The general aim of the work described in this thesis has been to elucidate the factors that affect the pathogenicity of *B. bassiana* towards *M. sexta* (i.e. its ability to cause disease) and the virulence towards its insect host once the fungus is established (i.e. its propensity to cause adverse symptoms and the speed with which these symptoms develop).

The metabolites from filtrates of three *B. bassiana* isolates, 1558, 2727 and 3527, grown *in vitro* significantly ($p<0.05$) affected the larval growth of *M. sexta* as evidenced by a dose-dependent reduction in weight gain. The filtrates also adversely affected the functions of larval haemocytes in combating microbial infection. These effects varied between fungal isolates as did the concentration of the putative immuno-suppressive factors. In this way my research has illuminated factors that are common to all pathogenic isolates, as well as those that vary between them.

5.2 Pathogenicity of *B. bassiana* isolates to *M. sexta* larvae

The use of two different methods of applying fungal spores, immersion and injection, enabled the identification of those isolates of *B. bassiana* which were able to penetrate the insect cuticle and continue to grow within the insect, as opposed to those which were only pathogenic if injected through the cuticle into the haemolymph.

The ability of an entomopathogenic fungus to penetrate the host insect depends on many factors. Some fungal isolates with low pathogenicity may have poor germination rates. Their lack of effect on the host may be due merely to their inability to germinate and commence appressorial formation on the cuticle surface. All twelve isolates tested were able to germinate on the cuticle surface and so this was unlikely to be a cause of the variation in their pathogenic success.

The fact that spores of those isolates that were ineffective after insect immersion (Isolates 959, 1122, 1315 and 1629) were nevertheless pathogenic after injection, suggests that inability to germinate was not a factor in their lack of pathogenicity. It is possible, however, that spores unable to germinate on the cuticle surface were stimulated into germination by the nutrient-rich haemolymph.

Some species of fungus are able to produce germinal tubes and directly penetrate their hosts, but *Beauveria bassiana*, like *Metarhizium anisopliae,* produces appressoria. This development may depend on the structure of the insect cuticle, or on elicitors that the fungus receives from the insect. Charnley (1984) showed that, in addition to chemical factors, the physical structure of the cuticle played an important part in appressorial formation by the fungus. The inability of four of the tested *B. bassiana* isolates to produce any mortality in the *M. sexta* larvae, even seven days after the immersion of the insect in spore suspensions, could be due to the inability of these fungal isolates to produce appressoria on the cuticle surface.

Pathogenic success of an isolate may be due not only to the ability of the spore to germinate rapidly, but also to the ability of the growing hyphae to adapt and utilise the nutrients within the environment. Hence those isolates that grow quickly may be able to kill their hosts by mycosis, or by the rapid production of toxins, or by a combination of both strategies.

A comparison of percentage mortality of larvae seven days after immersion (Table 4) with the LT_{50} of injected larvae (Table 3) shows that the isolates causing the lowest mortality after immersion also caused the longest LT_{50} after injection.

It may be that these are slow-growing isolates of *B. bassiana* and because of this the insects' defences are not overwhelmed and are able to prevent the fungus from developing and causing insect death.

5.3 Effects of *B. bassiana* on the growth of *M. sexta* larvae

In the work described in Chapter 2, it is shown that *B. bassiana* spores caused loss of weight gain and malaise in *M. sexta* larvae. Both treatment by injection and immersion in spore suspension affected the larvae, but the results were more pronounced after injection.

All twelve of the *B. bassiana* isolates compared came from lepidopteran hosts, but there was considerable variation in their effects on the larvae of the moth *M. sexta* . Some killed their hosts much more quickly (i.e caused a much shorter LT_{50}) than others, and whilst most were only effective by injection. All the isolates reduced larval weight gain in proportion to the concentration of inoculum. This was true whether the insects were immersed in, or inoculated with, spore suspensions.

The reduction in weight gain was also linked to a retarded onset of pupation. Fifth instar *M. sexta* larvae normally take 5 to 6 days to reach their "wandering" stage, when feeding and weight gain are reduced prior to pupation. They are unlikely to reach this stage of development at less than 8 g (Nijhont, 1975). Where fungal-treated larvae fed less and gained less weight, the wandering stage and subsequent pupation were delayed.

Undoubtedly, a number of different factors could contribute to this lack of weight gain. Where fungal spores penetrated the haemocoel and hyphal bodies, followed by mycelia, developed, the circulation of the haemolymph would be constricted. This would in itself impair the distribution of nutrients within the insect and depress its weight gain. However the fungus would also directly remove soluble nutrients from the insect's haemolymph, subverting them for its own growth. It is possible that the fungus may actually be able to interfere with the host's regulation of haemolymph composition, in order to gain nutrients. For example, Xia and Charnley (unpublished) have shown that *M. anisopliae* secretes glucosidase enzymes into the host insect's body fluids and thus increases the supply of monosaccharides to the fungus. Ultimately activities of this sort may lead to the death of the insect by "starvation".

Laminaran-like substances attached to the surface of spores might be responsible for the reduced weight gain of larvae. Laminarans are polysaccharides composed of β-(1-3) linked glucose residues. Yoshikawa (1983) noted that many fungi contain laminarans as major structural elements of their cell walls. These polysaccharides are heat-stable and can be extracted from mycelial walls by autoclaving.

They have been shown to be still active in eliciting plant responses even after heat treatment and thus are demonstrably heat stable. Dunn (1991) was able to show that cell wall fragments from the breakdown of bacterial cells by lysozyme were the causative agent of insect malaise. *Manduca sexta* larvae produced lysozyme in response to *E. coli* infection and larvae injected with peptidoglycan fragments from bacterial walls broken down with lysozyme exhibited a "malaise syndrome". This reduced insect feeding and weight gain and delayed metamorphosis. However, Boucias *et al.* (1994) was unable to stimulate the synthesis of lysozyme, a haemolymph protein, in *Spodoptera exigua* with *Beauveria bassiana* spores, which might be due to the difference in components of bacterial and fungal cell walls.

In the present work, experiments with autoclaved spores showed that heating destroyed any effect on weight gain and that therefore the dose-dependent reduction of growth was not due to heat-stable substances on the exterior of the spores.

Although in Dunn's 1991 experiments cell wall fragments from the break down of bacterial cells by lysozyme appear to be the causative agent of insect malaise, to date there is no published work to show that fungal wall fragments cause the same malaise syndrome. Fungal cell walls are composed chiefly of chitin, whilst bacterial walls are of peptidoglycan. Although both components can be split by the enzyme lysozyme to yield N-acetyl glucosamine, bacterial peptidoglycan also yields N-acetyl muramate. This may be responsible for the difference in insect reaction to bacterial and fungal cell walls.

Interestingly, injected filtrates from the different isolates also elicited a larval response of malaise and lack of weight gain which appeared to be dose-related. Since these filtrates were free of fungal spores it seems that the effect is probably due to toxic metabolites produced by the fungus *in vitro* and not to cell wall components. Although it is possible that remnants of peptidoglycan from the fungal cell wall were also dissolved in the filtrate and could affect larval weight gain, fungal toxins seem a more likely cause, since cell wall fragments from the different isolates would have affected weight gain similarly and this was not the case. The filtrates from different isolates of *B. bassiana* had very different effects on insect weight gain.

5.4 Effects of fungal metabolites on *M. sexta* larvae *in vivo*

B. bassiana is known to produce a wide range of metabolites toxic to insects. By examining the culture filtrate it was possible to distinguish between the action of the

fungal spore on the insect and the effect of metabolites produced during *in vitro* culture and passed into the media.

There exist numerous records of the toxicity of *B. bassiana* filtrates to insects, but actual identification of the toxins is less common. This may be due to the fact that isolates vary in their toxin productivity and host insects in their susceptibility.

Kucera and Samsinákóvá (1968) showed that *B. bassiana* produced at least two toxic proteases in the culture filtrate and that one was toxic by injection to *Galleria* larvae, thus linking the proteolytic and toxic activity. Samsináková *et al.* (1977) went on to show that *B. bassiana* isolates varied in their production of enzymes, but that Colorado beetle mortality correlated with the protease and chitinase activity of the fungus.

Although the two enzymes are essential for the first phase of fungal attack of the insect cuticle, Gardner *et al.* (1979) showed that *B. bassiana* was able to change the pattern of normal protein metabolism of larval *Spodoptera eridania* and *S. frugiperda* in the last stages of mycosis and it is possible that protein metabolism and enzyme synthesis are affected throughout the fungal infection of insects.

Beauvericin is one of the most widely produced metabolites of *B. bassiana*, though Champlin and Grula (1979) could not detect it in liquid cultures nor in the haemolymph of *Heliothis zea* larvae injected with *B. bassiana*. However, Genthner *et al.* (1994) showed that isolates of *B. bassiana* produced beauvericin *in vivo* which was toxic to the brine shrimp, *Mysidopsis bahia*. Although beauvericin is produced commercially by fermentation (Sigma Chemical Co., St Louis, MO) it seems probable that its production by the fungus depends on the particular isolate of *B. bassiana* and its cultural conditions. It also seems probable that it is produced by the mycelia and not released into aqueous media, so it is unlikely to be found in the filtrate.

Gupta *et al.* (1994) isolated a novel analogue of beauvericin from the mycelia of *B. bassiana* which was effective against the mosquito larvae, *Aedes aegypti*. Like the other forms of beauvericin it needed to be extracted from the mycelia with methanol and methylene chloride and so would not have passed into the culture filtrate.

Since the action of both the live spores and the sterile culture filtrate on *M. sexta* larvae was studied in the current work, if beauvericin was one of the toxic metabolites produced it would only be likely to occur where the spores were used and mycelia developed within the insect body. Beauvericin is produced by the mycelium and

does not normally pass through the cell membrane and so is unlikely to be found in the filtrate.

Bassionolide, a cyclodepsipeptide, has also been isolated from *B. bassiana* mycelia (Kanaoka *et al.* 1978) and shown to be effective against *Bombyx mori* larvae *per os* , causing atonic symptoms at 2 μg per larva. However, like beauvericin, bassionolide appears to be held by the mycelial membrane and so would be unlikely to pass into the culture filtrate. Additionally, none of the *M. sexta* larvae injected with the culture filtrate exhibited the paralysis which is associated with bassionolide.

Bassiatin is one of the most recently characterised products of *B. bassiana* and is similar in structure to beauvericin. Like beauvericin it does not appear to pass from the mycelia to the culture filtrate and has to be extracted from the mycelia with acetone and ethyl acetate (Kagamizono *et al.* 1995). So far bioassays of bassiatin have been restricted to rabbit platelet aggregation and its toxicity towards insects is unknown. Bassiatin may be yet another common metabolite of *B. bassiana*, but its presence in culture filtrates is unlikely and if it has affected *M. sexta* larvae it is more likely to have done this during fungal growth within the insect than by filtrate injection.

One of the few *B. bassiana* products which passes regularly into aqueous media was identified by Vining, Kelleher and Schwarting (1962) as oosporein. This red pigment is a dibenzoquinone produced by *Chaetomium trilaterale* (Cole *et al.* 1997) as well as by *Beauveria tenella* and *Beauveria bassiana.* Its production is variable with only some isolates of *B. bassiana* producing it and appears to depend on the media in which the fungus is grown. El Basyouni *et al.* (1968) showed that although some isolates of *B. bassiana* lost the ability to produce oosporein others retained this ability if grown on malt agar or Sabouraud maltose agar. He also showed that production of oosporein by *B. tenella* responded to increasing amounts of sodium nitrate up to a value of three grams per litre and was not even suppressed by amounts of 8 grams of sodium nitrate per litre. Additionally a higher production of oosporein was achieved with this fungus using glycerol instead of glucose as a carbon source in the medium.

Eyal *et al.* (1994) noted that some isolates lost their ability to produce the red pigment when transferred to agar and so he developed a method of storing *B. bassiana* mycelia on alginate beads. He showed that the production of oosporein was unaffected by storage in the mycelia-alginate beads, but that the pigment would not be produced without the support of agar or liquid medium.

Bioassays on whitefly showed that some oosporein-producing isolates of *B. bassiana* were also highly virulent against whitefly. Eyal suggested that oosporein may act as an antagonist to insect intestinal flora, thus allowing the fungal mycelia to develop faster, but although the pigment coloured the whitefly, toxicity was not demonstrated.

In the present work with *Manduca sexta,* the *B. bassiana* isolates varied considerably in their ability to produce a red pigment (presumed to be oosporein). Where isolates produced this pigment it passed easily into the liquid medium, but although it was obviously water-soluble it had no evident toxic effect on *M. sexta* larvae. There was no lack of weight gain or increased mortality which was attributable to the pigment, though it may have acted as an antagonist to gut flora and altered the insect growth rate. The effects of the pigment and other metabolites were not separated and other than the pink colouration of insects injected with pigment-producing isolates, there was no evident effect on the larvae.

Roberts (1980), in his article on the toxins of entomopathogenic fungi, suggests that oxalic acid could be an important toxin in the haemolymph of insects infected with *B. bassiana.*

Oxalic acid normally occurs in plants as ammonium oxalate. Cordon and Schwartz (1962) found that *B. brongniartii* converted 20% of the original media solids into oxalic acid, and both Kodaira (1961) and Müller-Kögler (1965) found oxalate crystals on the surface of insects killed by *B. bassiana.* Since *B. bassiana* is known to produce oxalic acid which is soluble in aqueous solutions it is probable that it is one of the major components in the filtrate after the liquid culture of the fungus.

The amount of oxalic acid found in the filtrate varied considerably between the isolates after 8 days growth in liquid Czapek-Dox. However, fifth instar larvae injected with doses of synthetic oxalic acid, at twice the concentration produced by the highest-yielding fungus, showed no significant difference in weight gain from the control. There were no noticeable pathogenic effects on the larvae which went on to pupate normally.

Oxalic acid production varied by a factor of ten between the fungal isolates, but concentration did not correlate with pathogenicity. Some isolates that produced large amounts of oxalic acid *in vitro,* for example 304, might have been successful at penetrating the insect cuticle, others producing equally high amounts (Isolate 1122), were not. Since cuticle penetration depended on spore germination and appressorial formation and oxalic acid is produced by the fungal mycelia there is little likelihood that

cuticle penetration and oxalic acid production are linked.

Oxalic acid production by fungal growth within the insect is likely to be buffered by the proteins of the haemolymph. Injections of relatively large amounts of artificial oxalic acid were tolerated by the insects and did not appear to have deleterious effects and it is probable that they would tolerate oxalic acid of fungal origin in the same way.

It would appear that the larvae were able to tolerate high levels of oxalic acid, and that the insect haemolymph was able to buffer its effects. Although the low pH caused by the oxalic acid on its own is unlikely to affect the insect it could chelate calcium ions and thus affect muscle contraction and nervous responses.

5.5 Response of *M. sexta* haemocytes to spores of different isolates of *B. bassiana*

It was evident on the examination of the haemolymph that there was considerable variation in the strategies of the different *B. bassiana* isolates. Haemocytes of *M. sexta* larvae recognised the invasion of fungal conidiospores and responded by either phagocytosis or aggregation. These conidiospores would not occur naturally in the insect haemocoel and Boucias and Pendland (1991b) considered that haemocytes of *Spodoptera exigua* recognized conidiospores of *B. bassiana* non-specifically. They considered, that the recognition of fungal blastospores, by contrast, was mediated by opsonins.

Blastospores of *B. bassiana* develop from mycelia in liquid culture, both *in vitro* and within the insect haemolymph. *In vivo* they are produced after the fungus has penetrated the insect cuticle and reached the haemocoel. The mycelia buds in a yeast-like manner and releases blastospores into the haemolymph. Hung and Boucias (1992) suggested that blastospores produced *in vivo* possessed a cell surface that was not recognized as foreign by *Spodoptera exigua* haemocytes. However, blastospores produced *in vitro* and injected into the larvae were recognized and rapidly phagocytosed by the haemocytes.

5.5.1 Phagocytosis

Even though the cell wall of conidiospores might not contain the same elicitors as blastospores, in the current work larval haemocytes were stimulated to phagocytose

conidiospores. The injection of blastospores instead of conidiospores might stimulate a greater response, but it is not certain that blastospores grown *in vitro* would have the same spore coat or elicitors as those developing *in vivo.*

Ratcliffe and Walters (1983) considered phagocytosis to be the primary cellular defence reaction of larval haemocytes to bacterial infection. They showed that phagocytosis was related to the pathogenicity of the bacteria and suggested that cell wall components were central to the insect recognition of infection. In their work injected *in vitro* blastospores were phagocytosed, whilst wall-less hyphal bodies were not recognised by immunocompetent haemocytes.

The current work shows clearly that phagocytosis of *B. bassiana* conidiospores *in vitro* only occurred at a low level, below 2% of the available spores, but that a factor in some fungal filtrates actively reduced its occurrence and had a suppressive action on cell aggregation. Thus any relationship of phagocytosis to pathogenicity was obscured by the immuno-suppressive effects of the fungal filtrates.

Insect haemocytes reacted to the presence of fungal spores by rounding up and withdrawing their filipodia. As might be expected, the number of haemocytes in infected insects dropped between 48 and 72 hours after treatment, but after treatment with two of the isolates, 959 and 1558, the number of haemocytes returned to the original level 96 hours after treatment. Neither of these isolates were very effective pathogens, and whether the rise in haemocyte count reflected the insect's ability to recover from fungal attack, or was the factor that prevented the development of blastospores within the haemocoel, the outcome was the same.

The haemocytes of *M. sexta* larvae may recognise the invasion of fungal spores of *B. bassiana* and respond by phagocytosing them. However, whilst blastospores and conidiospores may be engulfed, they are not necessarily broken down.

Pendland *et al.* (1993) showed that haemocytes in the larval haemolymph of *Spodoptera exigua* were able to phagocytose *B. bassiana* blastospores produced *in vitro.* Cell wall components appeared to be central to the insect recognition of infection since injected *in vitro* blastospores were phagocytosed, whilst wall-less hyphal bodies were not recognised by immunocompetent haemocytes.

In the present work, it was shown that larval haemocytes were able to recognise and phagocytose *Beauveria bassiana* conidiospores, but that this ability was reduced by the introduction of fungal culture filtrate. Although the conidiospores were

phagocytosed it was not clear that they were digested. Where monolayers were incubated for 24 hours and haemocytic nodules formed, some conidiospores were able to germinate and grow out of the engulfing nodule.

Even without the sophistication of some of the immune responses of vertebrates, phagocytosis is not the only response of insect haemocytes to infection.

5.5.2 Effect of spores of different isolates on larval haemocytic aggregation

Hung *et al.*, (1993) showed that haemocytes of *Spodoptera exigua* not only phagocytosed the yeast, *Candida albicans*, but were able to form multi-layered haemocytic nodules around the yeast cells. By contrast, blastospores of *B. bassiana* did not stimulate this nodule formation and were rapidly phagocytosed.

By using *B. bassiana* conidiospores, which may have had different elicitors from blastospores on the cell surface, the current work clearly showed that these spores stimulated nodule formation in *M. sexta*. Larval haemocytes responded to fungal infection, both *in vitro* and *in vivo*, but the response varied markedly between isolates. There was a distinct correlation between the pathogenicity of an isolate and the lack of nodule formation. The more pathogenic the isolate, in terms of percentage mortality and length of LT_{50} , the fewer the nodules formed. By contrast, the less pathogenic the isolate, the greater the number of haemocytic nodules formed both *in vivo* and *in vitro*.

To date there are no literature references to correlation of fungal pathogenicity with multi-cellular haemocytic nodule formation. In the current work, a significant correlation was found between nodule numbers and LT_{50}, thus clearly demonstrating a link between the nodular response of *M. sexta* larval haemocytes and the pathogenicity of *B. bassiana* conidiospores.

5.5.3 Immunosuppressive effects of fungal culture filtrates compared with eicosanoids

Although the effect on haemocytes of the culture filtrates appeared similar to the effects of eicosanoids, the active agent is likely to be quite different. Stanley-Samuelson (1994) in his review looked at the wide range of reported effects of eicosanoids on insect physiology. These included the regulation of the temperature set points in desert cicadas, the frequency of egg-laying in house crickets and the flight capabilities of the adult mosquito. His own work on insect immunity (Stanley-Samuelson 1991) showed that inhibition of eicosanoid synthesis lead to the inability of *Manduca sexta* larvae to clear bacterial infection. He concluded that eicosanoids were specifically involved in mediating cellular responses to bacterial infection and he was able to show that dexamethasone and indomethacin prevented the normal cellular response to bacterial infection *in vivo*. He went on to demonstrate that the response could be "rescued" by the injection of treated insects with the prostaglandin precursor arachidonic acid.

Miller *et al.* (1994) showed that nodule formation in the haemolymph of fifth instar *Manduca sexta* larvae infected with the bacteria *Serratia marcescens* was inhibited by intrahaemocylic injections of the eicosanoid dexamethasone. The number of nodules formed was reduced in a dose-related manner, but the subsequent injection of arachidonic acid, a prostaglandin precursor, reversed the effects of dexamethasone. The injection of palmitic acid which, unlike arachidonic acid, cannot be used by the insect to form prostaglandins, did not restore the ability of haemocytes to form nodules, thus demonstrating the importance of arachidonic acid as a precursor of prostaglandins.

In the current work *in vitro*, the culture filtrate from some of the isolates of *Beauveria bassiana* also inhibited the normal cellular response of larval haemocytes. It was appropriate to attempt to regain the aggregation response with arachidonic acid since this could show if the filtrate acted in the same way as eicosanoids in preventing the formation of prostaglandins from arachidonic acid.

The suppression of cell aggregation by fungal filtrates was not significantly altered by the addition of arachidonic acid to the monolayers. From the results obtained it was clear that a small protein produced by the culture of some of the isolates of *B. bassiana in vitro* was responsible for inhibiting haemocyte aggregation. This protein may not block prostaglandin synthesis, but act on the haemocytic response in a different way. Further work might show whether the protein itself acted on the cell membrane, or whether it was part of a cascade of events which prevented the normal response of insect haemocytes to infection. It is likely that several different mechanisms exist which trigger haemocyte aggregation and some of these mechanisms may only occur in the whole organism. Hampson *et al.* (1992) showed that the blood cells of the shore crab, *Carcinus maenas*, were able to synthesize prostaglandins *in vitro* and thus would be able to overcome anti-aggregation effects of phospholipase inhibitors like dexamethasone and indomethacin. It is possible that *M. sexta* haemocytes can also do this, but it seems more likely that the fungal culture filtrate affects an entirely different pathway from that of the phospholipase inhibitors, even though its effects on haemocyte aggregation are similar.

5.5.4 Immuno-suppressive components in the culture filtrate of *B. bassiana* isolates

Fractions of filtrates from the different isolates of *B. bassiana* were separated by passing them through membrane filter devices with a 10kDa pore size. The <10kDa fraction was then heated to 100°C for 20 minutes. It was shown that heat-treated

fractions containing only components smaller than 10kDa were still active in preventing haemocyte aggregation. The extent of suppression of this nodule formation was dependent on the dose of the fungal filtrate and invoked a response at considerable dilutions, but only occurred in the filtrates of some isolates.

SDS-PAGE gels of concentrated culture filtrate from different fungal isolates showed bands of a small protein (approx.6.5 kDa) occurring in the filtrates from immuno-suppressive isolates. These bands were absent in the isolate culture filtrates which did not have an effect on haemocyte aggregation suggesting that it is this small heat-stable protein which is responsible for the suppression of the haemocytic response.

5.6 Future work

The current work has provided information on the pathogenicity of different *B. bassiana* isolates towards *M. sexta*. However it is clear from the effects of the fungal filtrates that the pathogenicity is not entirely due to mycosis and that toxic elements from fungal growth also have considerable effects on the insects. The current work has added to an understanding of the way in which some fungal isolates are more successful pathogens than others.

The effects of fungal culture filtrates, both from *B. bassiana* and other entomopathogens, need to be assessed on other insect species. *M. sexta* is a useful model insect since its size is convenient and laboratory management not difficult, but the effects of these fungal toxins on other insect species need to be established.

The mechanism with which the fungal metabolites are able to overcome the haemocytic response may be limited to a few isolates, or it may be widespread but only evident in isolates which can first penetrate the insect cuticle. The immuno-suppressive mechanism increases the speed with which the target insect is affected. Since the effects are quicker than mycosis, isolates producing this active agent would cause a shorter LT_{50} for susceptible insects and be more useful pathogens.

The small protein which appears to be responsible for the immuno-suppressive effect needs to be recovered from a native gel (without SDS) and assessed for activity against larval haemocytes. It should also be possible to obtain a peak for this protein using HPLC. The appropriate fraction could then be collected, characterised and tested for its anti-coagulation effects on larval haemocytes. Additionally, antibodies could be raised to the protein, which could then be accurately characterized and should provide a

purified substance suitable for the further studies on insect larval immuno-suppression.

Even though the active agent against haemocytic aggregation has been demonstrated in fungal filtrates it is unlikely to be a useful agent for insect pest control in the field. The only way in which it could be introduced into the insect haemocoel on a large scale would be by isolate selection or genetic modification to produce fungal isolates which could both penetrate the insect cuticle and produce the anti-aggregation agent within the insect. Before fungal isolates with these qualities could be released for insect-pest control, extensive trials would need to be undertaken to ensure that beneficial and non-harmful insects were not targeted together with the insect pest.

Mycotoxins are some of the most powerful poisons known to man and their release for the control of field crop pests could cause untold damage to other living organisms. The effects on other species and systems of these toxins and their breakdown products are as yet poorly researched, and toxicological problems could ultimately preclude their direct use as a safe method of insect pest control. Nevertheless, better understanding of the nature of the interactions between insect host and fungal pathogen must be useful in improving the utility of natural or genetically manipulated isolates of entomopathogenic fungi for biological control of insect pests.

APPENDIX I

Source of Isolates

Isolates of *Beauveria bassiana* obtained from the United States Department of Agriculture's collection held at the Boyce-Thompson Institute for Plant Research (Ithaca, N.Y., U.S.A.).

Isolate No.	Insect Host	Origin
304	*Bombyx mori*	Japan, 1967.
959	*Spodoptera frugiperda*	Brazil, 1983.
1007	*Bombyx mori*	Japan, 1983
1122	*Cydia pomonella*	Italy, 1983.
1315	*Helicoverpa virescens*	France, 1984.
1484	*Diatraea saccharalis*	Brazil, 1984.
1558	*Cossula cossus*	Italy, 1981.
1629	Lepidoptera: *Noctuidae*	Hungary, 1984.
1886	*Chilo infuscatellus*	India, 1985.
2417	*Emmalocera depressella*	India, 1987.
2727	*Plutella xylostella*	Phillipines, 1989.
3527	*Lymantria dispar* (6L)	U.S.A.,1991.

APPENDIX II

Artificial diet for *Manduca sexta* larvae.

Premix :-

Wheat germ	750 g
Casein	350 g
Sucrose	300 g
Dry yeast	150 g
Wesson's salt mixture	100 g
Sorbic acid	15 g
Cholesterol	10 g
Methyl-p-hydroxybenzoate	10 g
Choline chloride	10 g

Additions :-

Ascorbic acid	8 g
Aureomycin	0.2 g
Vanderzant's vitamin mix	0.2 g
10% Formaldehyde	5 ml
Raw linseed oil	4 ml
Maize oil	4 ml
Agar	25 g
Distilled water	1.7 l

Preparation

336 grams of the pre-mix is put in a Waring blender with 700 ml of boiling distilled water. The agar is heated separately with 1000 ml of distilled water until boiling when it is added to the mix. When the mix temperature has dropped to 70° C the oils, formaldehyde, vitamins, ascorbic acid and aureomycin are added.. After mixing, the diet is poured into foil-lined trays and cooled in a flow cabinet, then stored at 4° C.

APPENDIX III

Media for fungal cultures

Sabouraud's Dextrose Agar (SDA)

Dextrose	40 g
Mycological peptone	10 g
Agar (No.3)	20 g
Yeast extract	5 g
D. H_2O	1 litre

Mix and heat to dissolve, adjust pH to 6.5 with sodium hydroxide.

Sterilise at 15 psi for 10 min.

Optionally add antibiotics when temperature below 60° C.

0.5 g per litre cyclohexamide (Sigma)

0.6 g per litre chloramphenicol

Czapek-Dox liquid medium (modified)

Dissolve 33.4 g of Czapek-Dox with 5 g of bacteriological peptone in 1 litre of distilled water. Mix well and adjust pH to 6.5. Sterilise by autoclaving for 20 min at 10 psi.

APPENDIX IV

Buffers used for haemolymph work

Anticoagulant buffer

0.098 M	NaOH	Sodium Hydroxide
0.146 M	NaCl	Sodium Chloride
0.017 M	EDTA	Ethylenediaminetetraacetic Acid
0.041 M		Citric acid

In 1 litre of endotoxin-free water.

pH 4.5 Osmolality 370 mOs kg^{-1}

Autoclaved at 120° C for 20 minutes.

Carbonate-bicarbonate buffer

(for rhodamine work).

100 ml 0.2M	Na_2CO_3	Sodium Carbonate
500 ml 0.2M	$NaHCO_3$	Sodium Bicarbonate

pH 9

Autoclaved at 120° C for 20 minutes

APPENDIX V

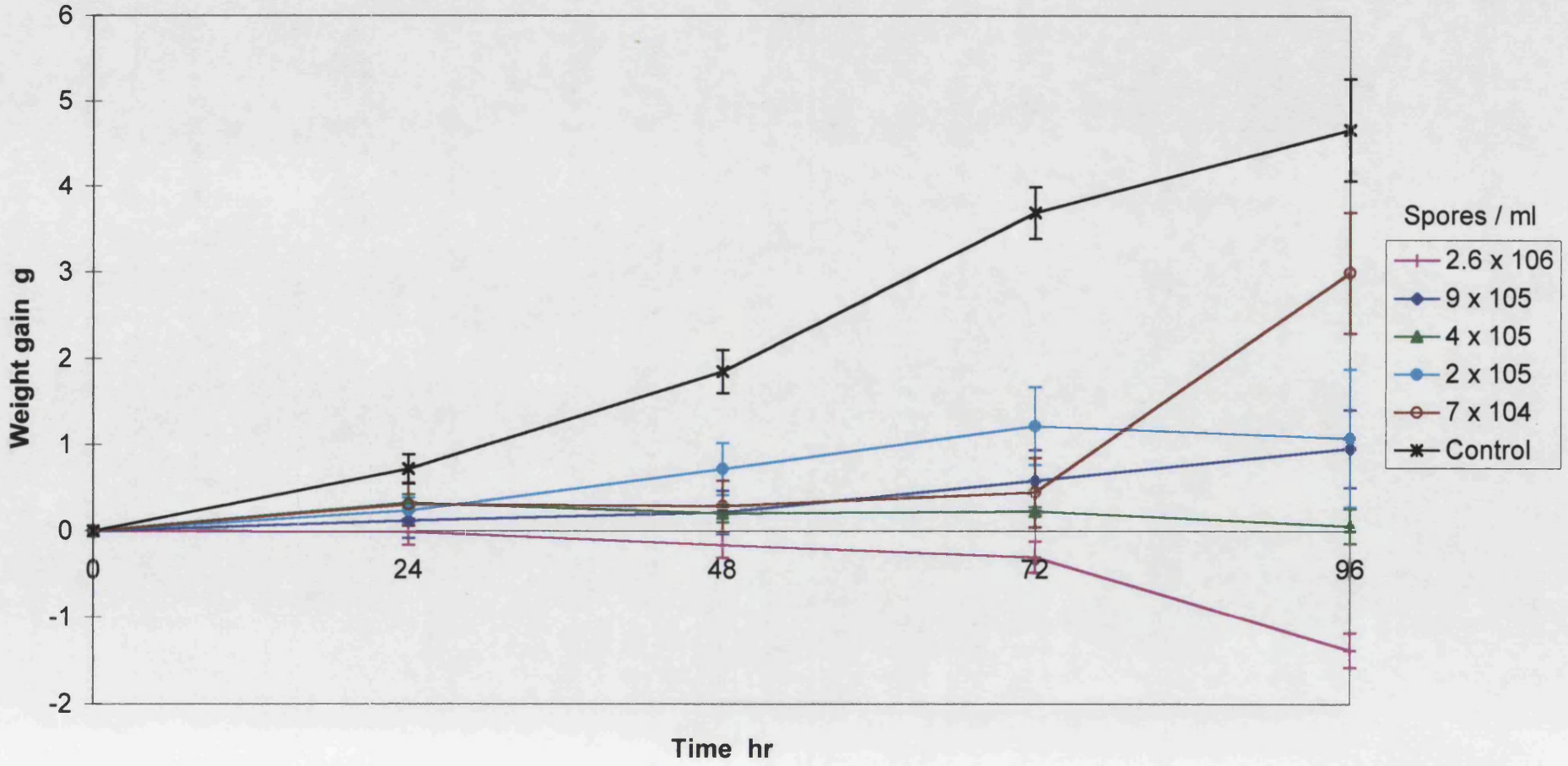
The figures on the following three pages show the weight gain of fifth instar *Manduca sexta* larvae over 5 days after the injection of conidiospores of different isolates of *Beauveria bassiana.*

Five first day fifth instar larvae were weighed and then injected with 10μl of spore suspensions of isolate 304,1315, or 3527. Four different conidial concentrations in distilled water were used for each isolate and five larvae injected in each case. The larvae were incubated at 25°C and weighed at 24 hour intervals.

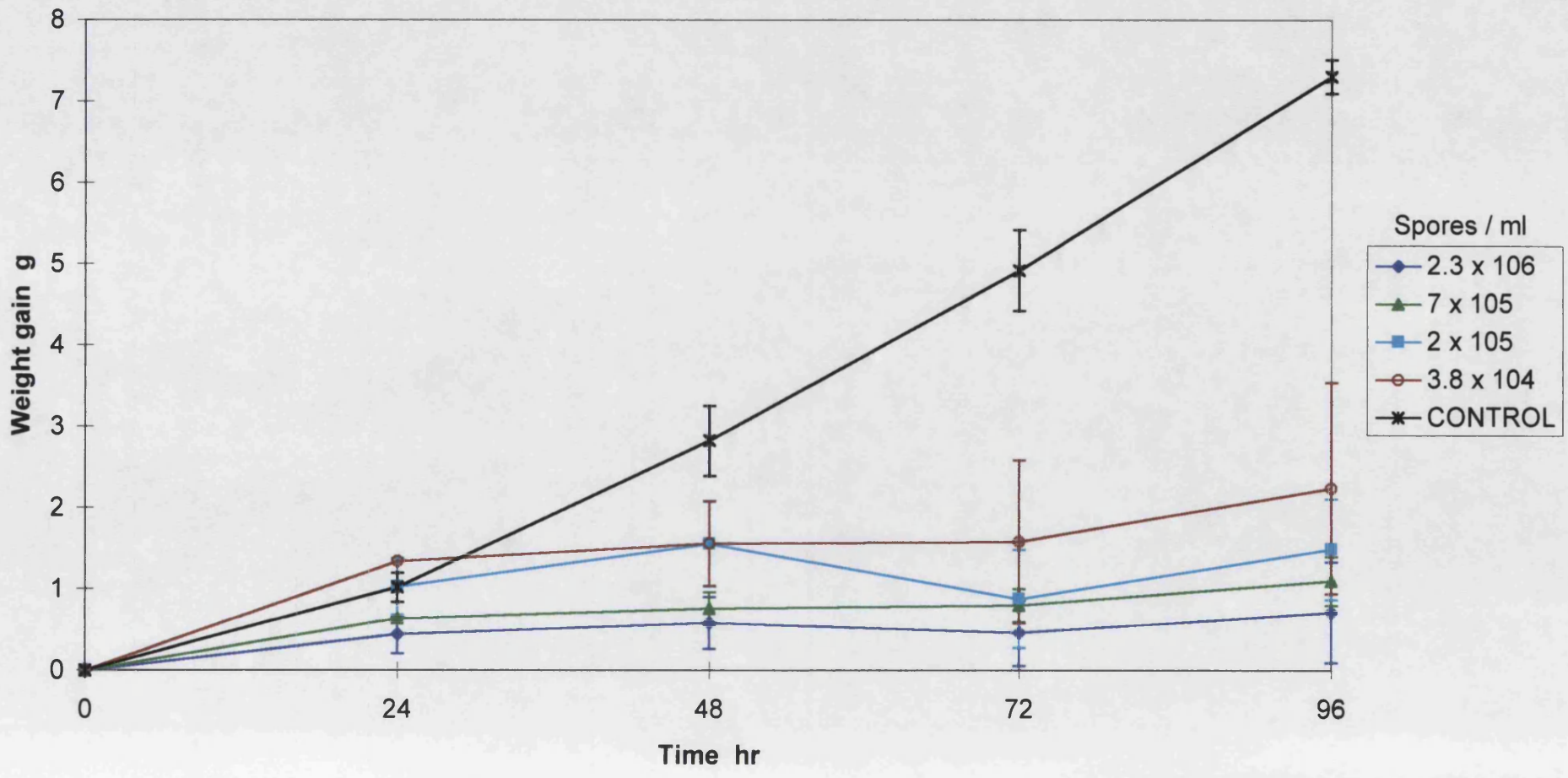
Control insects were injected with sterile distilled water.

The work was repeated three times with five larvae for each conidial concentration giving n = 15.

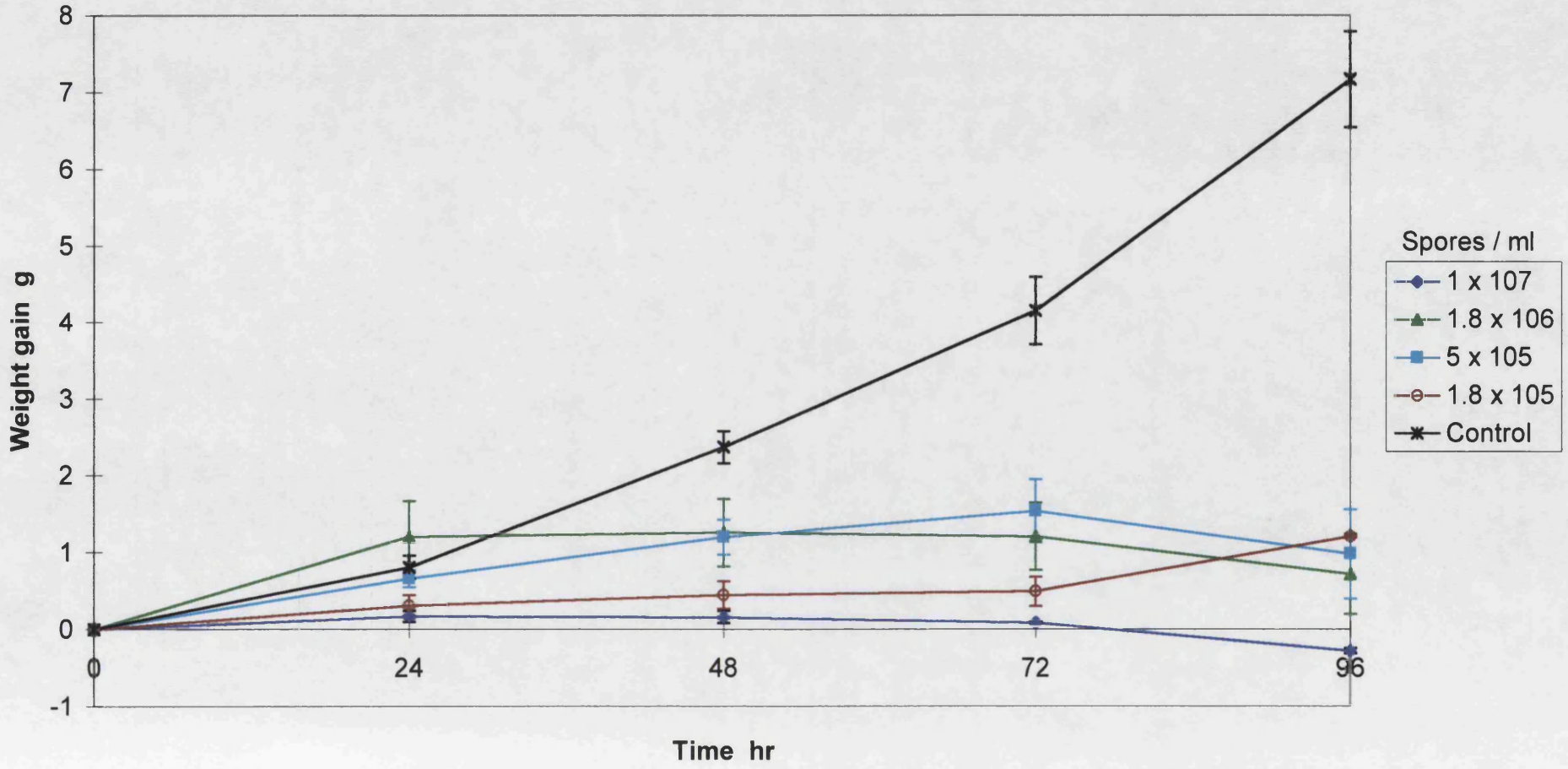
APPENDIX V - A. Larvae injected with 10µl of Isolate 304



APPENDIX V - E. Larvae injected with 10µl of Isolate 1315



APPENDIX V - J. Larvae injected with 10µl of Isolate 3527



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